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(54) Title: A TRANSCRIPTION FACTOR

(57) Abstract

A transcription factor regulating α-amylase promoter initiated expression in filamentous fungi, especially in *Aspergillii*, DNA sequences coding for said factor, its transformation into and expression in fungal host organisms, and the use of said factorin such hosts for increasing the expression of a polypeptide of interest being produced by said host.

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Title: A Transcription Factor

FIELD OF THE INVENTION

The present invention relates to a transcription factor found in filamentous fungi, especially in *Aspergillii*, DNA sequences coding for said factor, its transformation into and expression in fungal host organisms, and the use of said factor in such hosts for increasing the expression of a polypeptide of interest being produced by said host.

BACKGROUND OF THE INVENTION

Transcription factors are well known proteins involved in the initiation of transcription. They have been studied intensively in many different organisms and have also been described in fungi. Dhawale and Lane (NAR (1993) **21** 5537-5546) have recently compiled the transcription factors from fungi, including the filamentous fungi.

Many of the transcription factors are regulatory proteins; they bind to the promoter DNA and either activate or repress transcription as a response to stimuli to the cell.

The expression of the α-amylase gene in *A. oryzae* is regulated in response to the available carbon source. The gene is expressed at its maximum when the organism is grown on starch or maltose (Lachmund *et al.* (1993) *Current Microbiology* **26** 47-51; Tada *et al.* (1991) *Mol. Gen. Genet.* **229** 301-306). The expression of α-amylase is regulated at the transcriptional level as shown by Lachmund *et al.* (*supra*), which strongly suggests that transcription factors are involved in the regulation, but so far no gene for such a factor has been identified.

The promoter of the α-amylase gene has been studied by deletion analysis (Tada *et al.* (1991) *Agric. Biol. Chem.* **55** 1939-1941;

Tsuchiya *et al.* (1992) *Biosci. Biotech. Biochem.* **56** 1849-1853; Nagata *et al.* (1993) *Mol. Gen. Genet.* **237** 251-260). The authors of these papers propose that a specific sequence of the promoter is responsible for the maltose induction. Nagata *et al.* (*supra*) used this sequence as a probe in a gel shift experiment to see whether any proteins from *A. nidulans* nuclear extracts were able to bind to the promoter sequence. Three such proteins were found, but no involvement of these proteins in expression was shown. None of the proteins have been purified or identified by other means. Their genes likewise remain unknown.

SUMMARY OF THE INVENTION

The present invention relates to a transcription factor regulating the expression of the α-amylase promoter in filamentous fungi.

Accordingly, in a first aspect the invention relates to a DNA construct comprising a DNA sequence encoding a transcription factor of the invention, which DNA sequence comprises

a) the transcription factor encoding part of the DNA sequence cloned into plasmid pToC320 present in *E. coli* ToC1058, DSM 10666, or

b) an analogue of the DNA sequence defined in a), which

i) is at least 60% homologous with the DNA sequence defined in a), or

ii) hybridizes with the same nucleotide probe as the DNA sequence defined in a), or

iii) encodes a transcription factor which is at least 50% homologous with the transcription factor encoded by a DNA sequence comprising the DNA sequence defined in a), or

iv) encodes a transcription factor which is immunologically reactive with an antibody raised against the purified transcription factor encoded by the DNA sequence defined in a), or

v) complements the mutation in ToC879, *i.e.* enables ToC879 to grow on cyclodextrin and produce lipase when transformed with said DNA sequence.

The full length genomic DNA sequence encoding a transcription factor has been derived from a strain of the filamentous fungus *Aspergillus oryzae* and has been cloned into plasmid pToC320 present in *E. coli* ToC1058, DSM 10666.

Said transcription factor encoding DNA sequence harboured in pToC320, DSM 10666, is believed to have the same sequence as that presented in SEQ ID NO: 1 and SEQ ID NO: 2. Accordingly, whenever reference is made to the transcription factor encoding part of the DNA sequence cloned into plasmid pToC320 present in DSM 10666 such reference is also intended to include the transcription factor encoding part of the DNA sequence presented in SEQ ID NO: 1 and SEQ ID NO: 2.

Accordingly, the terms "the transcription factor encoding part of the DNA sequence cloned into plasmid pToC320 present in DSM 10666" and "the transcription factor encoding part of the DNA sequence presented in SEQ ID NO: 1 and SEQ ID NO: 2" may be used interchangeably.

In further aspects the invention provides an expression vector harbouring the DNA construct of the invention, a cell comprising said DNA construct or said expression vector and a method of producing a peptide exhibiting transcription factor activity, which method comprises culturing said cell under conditions permitting the production of the transcription factor.

Such a transcription factor of the invention will typically originate from a filamentous fungus.

The term "filamentous fungus" is intended to include the groups Phycomycetes, Zygomycetes, Ascomycetes, Basidiomycetes and fungi imperfecti, including Hyphomycetes such as the genera *Aspergillus*, *Penicillium*, *Trichoderma*, *Fusarium* and *Humicola*.

The invention also relates to a method of producing a filamentous fungal host cell comprising the introduction of a DNA

fragment coding for any such factor into a filamentous fungus wherein an α-amylase promoter or a co-regulated promoter regulates the expression of a polypeptide of interest in a manner whereby said factor will be expressed in said fungus.

In a further aspect the invention relates to a method of producing a polypeptide of interest, the expression of which is regulated by an α-amylase promoter or a co-regulated promoter, comprising growing a filamentous fungal host cell as described above under conditions conducive to the production of said factor and said polypeptide of interest, and recovering said polypeptide of interest.

Finally the invention relates to the use of said factor for regulating the expression of a polypeptide of interest in a filamentous fungus.

In this context, regulation means to change the conditions under which the factor of the invention is active. This could mean different pH, substrate, etc. regimes, whereby the resulting effect is an improved regulation of the expression of the protein of interest.

Furthermore, regulation also comprises events occurring in the growth phase of the fungus during which the transcription factor is active. Depending on the circumstances, both advancing and/or postponing the phase wherein the factor is active may enhance the expression and thus the yield.

In addition, using standard procedures known in the art, the specific DNA sequences involved in the binding of a transcription factor may be identified, thereby making it possible to insert such sequences into other promoters not normally regulated by the factor and enabling those promoters to be under the regulation of said factor.

BRIEF DESCRIPTION OF THE TABLES AND DRAWING

In the figures

Fig. 1 shows the structure of the plasmid pMT1657, the construction of which is described in Example 1;

Fig. 2 shows the structure of the plasmid pToC316, the construction of which is described in Example 1;

Fig. 3 shows the structure of the plasmid pToC320, the construction of which is described in Example 1;

Fig. 4 shows the structure of the plasmids pToC342 and pToC359, the construction of which are described in Example 3;

Fig. 5 shows the structure of the plasmid pToC298, the construction of which is described in Example 4;

Fig. 6 shows the results of lipase production by a p960 transformant of *A. oryzae* IFO4177 cultured in YP media containing 2% glucose (—■—) or 10% glucose (—◆—), in comparison to ToC1075 cultured in YP media containing 2% glucose (—□—) or 10% glucose (—◇—) and described in Example 4;

Fig. 7 shows the results of lipase production by ToC1139 cultured in YP media containing 2% glucose (—■—) or 10% glucose (—◆—), in comparison to ToC1075 cultured in YP media containing 2% glucose (—□—) or 10% glucose (—◇—) and described in Example 4; and

Fig. 8 shows the autoradiograph results of *A. niger* DNA digested with the following restriction enzymes: lane 2, *XbaI*; lane 3, *XmaI*; lane 4, *SalI*; lane 5, *HindIII*; lane 6, *EcoRI*; lane 7, *BglII*; lane 8, *BamHI*; lanes 1 and 9 contain ^{32}P-labelled 1 DNA digested with *BstEII*. The experiment is described in Example 5.

DETAILED DESCRIPTION OF THE INVENTION

In a first aspect the invention relates to a DNA construct comprising a DNA sequence encoding a transcription factor regulating an α-amylase promoter, which DNA sequence comprises

a) the transcription factor encoding part of the DNA sequence cloned into plasmid pToC320 present in *E. coli* ToC1058, DSM 10666, or

b) an analogue of the DNA sequence defined in a), which

i) is at least 60% homologous with the DNA sequence defined in a), or

ii) hybridizes with the same nucleotide probe as the DNA sequence defined in a), or

iii) encodes a transcription factor which is at least 50% homologous with the transcription factor encoded by a DNA sequence comprising the DNA sequence defined in a), or

iv) encodes a transcription factor which is immunologically reactive with an antibody raised against the purified transcription factor encoded by the DNA sequence defined in a), or

v) complements the mutation in ToC879, *i.e.* enables ToC879 to grow on cyclodextrin and produce lipase when transformed with said DNA sequence.

As defined herein, a DNA sequence analogous to the transcription factor encoding part of the DNA sequence cloned into plasmid pToC320 present in *E. coli* ToC1058, DSM 10666, is intended to indicate any DNA sequence encoding a transcription factor regulating an α-amylase promoter, which transcription factor has one or more of the properties cited under (i)-(v) above.

The analogous DNA sequence may be isolated from a strain of the filamentous fungus *A. oryzae* producing the transcription factor, or another or related organism and thus, *e.g.* be an allelic or species variant of the transcription factor encoding part of the DNA sequence cloned into plasmid pToC320 present in DSM 10666.

Alternatively, the analogous sequence may be constructed on the basis of the DNA sequence presented as the transcription factor encoding part of SEQ ID NO: 1 and SEQ ID NO: 2, *e.g.* be a subsequence thereof, and/or by introduction of nucleotide substitutions which do not give rise to another amino acid sequence of the transcription factor encoded by the DNA sequence, but which corresponds to the codon usage of the host organism intended for production of the transcription factor, or by introduction of nucleotide substitutions which may give rise to a different amino acid sequence.

When carrying out nucleotide substitutions, amino acid residue changes are preferably of a minor nature, that is conservative amino acid residue substitutions that do not significantly affect the folding or activity of the protein, small deletions, typically of one to about 30 amino acid residues; small amino- or carboxyl-terminal extensions.

Examples of conservative substitutions are within the group of basic amino acids (such as arginine, lysine, histidine), acidic amino acids (such as glutamic acid and aspartic acid), polar amino acids (such as glutamine and asparagine), hydrophobic amino acids (such as leucine, isoleucine, valine), aromatic amino acids (such as phenylalanine, tryptophan, tyrosine) and small amino acids (such as glycine, alanine, serine, threonine, methionine). For a general description of nucleotide substitution, see *e.g.* Ford, *et al.*, (1991), Protein Expression and Purification 2, 95-107.

It will be apparent to persons skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active transcription factor. Amino acid residues essential to the activity of the transcription factor encoded by a DNA construct of the invention, and therefore preferably not subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning

mutagenesis (cf. *e.g.* Cunningham and Wells, (1989), *Science* **244** 1081-1085). In the latter technique mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity (*i.e.* transcription factor regulating an α-amylase promoter) to identify amino acid residues that are critical to the activity of the molecule.

The homology referred to in (i) above is determined as the degree of identity between the two sequences indicating a derivation of the one sequence from the other. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., (1970), *Journal of Molecular Biology* **48** 443-453). Using GAP with the following settings for DNA sequence comparison: GAP creation penalty of 5.0 and GAP extension penalty of 0.3, the coding region of the DNA sequence exhibits a degree of identity preferably of at least 60%, more preferably at least 70%, more preferably at least 80%, more preferably at least 90%, more preferably at least 95% with the transcription factor encoding part of the DNA sequence shown in SEQ ID NO: 1 and SEQ ID NO: 2.

The hybridization referred to in (ii) above is intended to indicate that the analogous DNA sequence hybridizes to the same probe as the DNA sequence encoding the transcription factor under certain specified conditions, which are described in detail in the Materials and Methods section hereinafter. The oligonucleotide probe to be used is the DNA sequence corresponding to the transcription factor encoding part of the DNA sequence shown in SEQ ID NO: 1 or SEQ ID NO: 2 or a fragment thereof.

The homology referred to in (iii) above is determined as the degree of identity between the two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., *supra*). Using GAP with the

following settings for transcription factor sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1, the transcription factor encoded by an analogous DNA sequence exhibits a degree of identity preferably of at least 50%, more preferably at least 60%, more preferably at least 70%, even more preferably at least 80%, especially at least 90% with the transcription factor encoded by a DNA construct comprising the transcription factor encoding part of the DNA sequence shown in SEQ ID NO: 2, e.g. with the amino acid sequence SEQ ID NO: 3.

In connection with property (iv) the immunological reactivity may be determined by the method described in the Materials and Methods section hereinafter.

In relation to the property (v) the complementation method is described in Example 1 herein.

The DNA sequence encoding a transcription factor of the invention can be isolated from the strain *Aspergillus oryzae* IFO 4177 using standard methods *e.g.* as described by Sambrook, *et al.*, (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Lab.; Cold Spring Harbor, NY.

General RNA and DNA isolation methods are also disclosed in WO 93/11249 and WO 94/14953, the contents of which are hereby incorporated by reference. A more detailed description of the complementation method is given in Example 1 herein.

Alternatively, the DNA encoding a transcription factor of the invention may, in accordance with well-known procedures, be conveniently isolated from a suitable source, such as any of the below mentioned organisms, by use of synthetic oligonucleotide probes prepared on the basis of a DNA sequence disclosed herein. For instance, a suitable oligonucleotide probe may be prepared on the basis of the transcription factor encoding part of the nucleotide sequences presented as SEQ ID NO: 1 or any suitable subsequence thereof, or on the basis of the amino acid sequence SEQ ID NO: 3.

The invention relates specifically to a transcription factor regulating the expression of the α-amylase promoter in a filamentous fungus, which factor as indicated in Example 2 may even regulate the expression of other genes.

In this context the expression "filamentous fungus" is intended to include the groups Phycomycetes, Zygomycetes, Ascomycetes, Basidiomycetes and fungi imperfecti, including Hyphomycetes such as the genera *Aspergillus*, *Penicillium*, *Trichoderma*, *Fusarium* and *Humicola*.

In this context the expression "α-amylase promoter" means a sequence of bases immediately upstream from an α-amylase gene which RNA polymerase recognises and binds to promoting transcription of the gene coding for the α-amylase.

As indicated, transcription factors are known from many organisms and it is therefore expected that similar or corresponding factors may be found originating from other fungi of the genera *Aspergillus*, *Trichoderma*, *Penicillium*, *Fusarium*, *Humicola*, etc., having an enhancing effect on the expression of a polypeptide being under the regulation of amylase promoters in any fungus belonging to any of these genera.

A comparison of the DNA sequence coding for the transcription factor regulating the α-amylase promoter has shown some degree of homology to a transcription factor (CASUCI) regulating the expression of glucosidase in *Candida* and to MAL63 of *Saccharomyces cerevisiae* as disclosed in Kelly and Kwon-Chung, (1992) *J. Bacteriol.* **174** 222-232.

It is at present contemplated that a DNA sequence encoding a transcription factor homologous to the transcription factor of the invention, *i.e.* an analogous DNA sequence, may be obtained from other microorganisms. For instance, the DNA sequence may be derived by a similar screening of a cDNA library of another

microorganism, such as a strain of *Aspergillus*, *Saccharomyces*, *Erwinia*, *Fusarium* or *Trichoderma*.

An isolate of a strain of *A. oryzae* from which the gene coding for a transcription factor of the invention has been inactivated has been deposited by the inventors according to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure at the DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, DEUTSCHLAND.

Deposit date : 6 MAY 1996 (06.05.96)
Depositor's ref. : ToC879 = NN049238
DSM designation: *Aspergillus oryzae* DSM No.10671

The deposited strain *Aspergillus oryzae* DSM No.10671 can be used to isolate a transcription factor according to the invention from any strain of *Aspergillus oryzae* and any other fungal strain having such a gene by complementation as described hereinafter.

The expression plasmid pToC320 comprising the full length genomic DNA sequence encoding the transcription factor of the invention has been transformed into a strain of *E. coli* resulting in the strain ToC1058, which has been deposited by the inventors according to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure at the DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH., Mascheroder Weg 1b, D-38124 Braunschweig, DEUTSCHLAND.

Deposit date : 6 MAY 1996 (06.05.96)
Depositor's ref. : ToC1058 = NN049237
DSM designation: *E. coli* DSM No.10666

According to the invention, factors of this type originating from the species *A. oryzae*, *A. niger*, *A. awamori*, etc., especially *A. oryzae* IFO4177 are preferred.

The transcription factor of the invention has been found not only to be involved in the regulation of the α-amylase promoter, but also in the regulation of the glucoamylase promoter from *A. oryzae*.

Especially, the invention comprises any factor having an amino acid sequence comprising one or more fragments or combinations of fragments of the amino acid sequence depicted as SEQ ID NO: 3.

Truncated forms of the transcription factor may also be active. By truncated forms are meant modifications of the transcription factor wherein N-terminal, C-terminal or one or more internal fragments have been deleted.

A further aspect of the invention relates to a DNA sequence coding for any of these factors.

In this aspect the invention especially comprises any DNA sequence coding for one or more fragments of the amino acid sequence depicted as SEQ ID NO: 3.

More specifically the invention relates to a DNA sequence comprising one or more fragments or a combination of fragments of the DNA sequence depicted as SEQ ID NO: 1 and SEQ ID NO: 2.

According to a further aspect the invention relates to a method of producing a filamentous fungal host cell comprising the introduction of any of the above mentioned DNA fragments into a filamentous fungus wherein the α-amylase promoter or another co-regulated promoter regulates the expression of a polypeptide of interest in a manner whereby said factor will be expressed in said fungus.

The introduction of said DNA fragment may be performed by means of any well known standard method for the introduction of DNA

into a filamentous fungus, such as by use of an expression vector and host cells as described below.

Therefore, the invention also provides a recombinant expression vector comprising the DNA construct of the invention.

The expression vector of the invention may be any expression vector that is conveniently subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced.

Thus, the vector may be an autonomously replicating vector, *i.e.* a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.* a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the expression vector, the DNA sequence encoding the transcription factor should either also contain the expression signal normally associated with the transcription factor or should be operably connected to a suitable promoter and terminator sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes that are either homologous or heterologous to the host cell. The procedures used to ligate the DNA sequences coding for the transcription factor, the promoter and the terminator, respectively, and to insert them into suitable vectors are well known to persons skilled in the art (cf., Sambrook, *et al.*, *supra*).

Examples of suitable promoters for use in filamentous fungal host cells are, for instance, the A. *nidulans* ADH3 promoter (McKnight, *et al.* (1985) *The EMBO J.* **4** 2093-2099) or the *tpiA* promoter. Examples of other useful promoters are those derived from the gene encoding *Aspergillus oryzae* α-amylase, *Aspergillus niger* neutral a-amylase, *Aspergillus niger* acid stable a-

amylase, *Aspergillus niger*, *Aspergillus awamori*, or *Aspergillus oryzae* glucoamylase (*gluA*), *A. oryzae* alkaline protease (*alp*), *A. oryzae* nitrate reductase (*niaD*), *Aspergillus oryzae* triose phosphate isomerase (*tpi*), *Aspergillus nidulans* acetamidase, or an *Aspergillus* promoter coding for an amino acid biosynthetic gene such as *argB*.

In yet another aspect the invention provides a host cell comprising the DNA construct of the invention and/or the recombinant expression vector of the invention.

Preferably, the host cell of the invention is a eukaryotic cell, in particular a fungal cell such as a yeast or filamentous fungal cell. In particular, the cell may belong to a species of *Trichoderma*, preferably *Trichoderma harzianum* or *Trichoderma reesei*, or a species of *Aspergillus*, most preferably *Aspergillus oryzae* or *Aspergillus niger*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. The use of *Aspergillus* as a host microorganism is described in EP 238 023 (Novo Nordisk A/S), the contents of which are hereby incorporated by reference. The host cell may also be a yeast cell, *e.g.* a strain of *Saccharomyces*, in particular *Saccharomyces cerevisiae*, *Saccharomyces kluyveri* or *Saccharomyces uvarum*, a strain of *Schizosaccharomyces* sp., such as *Schizosaccharomyces pombe*, a strain of *Hansenula* sp., *Pichia* sp., *Yarrowia* sp., such as *Yarrowia lipolytica*, or *Kluyveromyces* sp., such as *Kluyveromyces lactis*.

The endogenous *amyR* gene of the host cell may be deleted or inactivated by other means. The introduction of *amyR* control by a heterologous promoter will then lead to a completely new scheme of regulation of the a-amylase promoter. If, for example, *amyR* is fused to the *A. oryzae niaD* promoter, the a-amylase promoter will become inducible by nitrate. If, instead of the *niaD* promoter, a *palC*-regulated promoter is used, the activity of the a-amylase promoter will be regulated by pH.

The invention also comprises a method of producing a polypeptide of interest, whereby a host cell as described above is grown under conditions conducive to the production of said factor and said polypeptide of interest, and said polypeptide of interest is recovered.

The medium used to culture the transformed host cells may be any conventional medium suitable for growing the host cells in question. The expressed polypeptide of interest may conveniently be secreted into the culture medium and may be recovered therefrom by well-known procedures including separating the cells from the medium by centrifugation or filtration, precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

According to the invention the method may be used to produce a polypeptide of interest that is a medicinal polypeptide, especially such medicinal polypeptides as growth hormone, insulin, blood clotting factor, and the like.

The method of the invention may also be used for the production of industrial enzymes, such as proteases, lipases, amylases, glucoamylases, oxido reductases, carbohydrases, carbonyl hydrolases, cellulases, esterases, etc.

According to a further aspect of the invention said transcription factor may be used for enhancing the expression of a polypeptide of interest in a filamentous fungus, such as a fungus of the genus *Aspergillus*, *Trichoderma*, *Penicillium*, *Fusarium*, *Humicola*, etc., especially of the species *A. oryzae*, *A. niger*, *A. awamori*, etc., and specifically *A. oryzae*.

The transcription factor of the invention may thus be used to enhance the expression of a medicinal polypeptide, such as growth hormone, insulin, blood clotting factor, etc.

Also, the expression of industrial enzymes, such as proteases, lipases, amylases, glucoamylases, oxido reductases, carbohydrases, carbonyl hydrolases, cellulases, esterases, etc., may be enhanced by the use of the transcription factor of the invention.

The transcription factor may also be used to identify the sequences in the a-amylase promoter to which it binds. For example, this could be done by making a GST-fusion protein with the DNA binding domain of AmyR, such as the zinc finger, for production in *E. coli*. Such fusion proteins may be conveniently made using commercially available kits, for example, "The GST Gene Fusion Kit" from Pharmacia. The purified GST-fusion protein can then be used in conventional *in vitro* techniques such as gel shift assays or DNA footprint analyses (Kulmburg, P., *et al.* (1992) Molecular and Cellular Biology **12** 1932-1939; Lutfiyya, L.L., and Johnston, M. (1996) Molecular and Cellular Biology **16** 4790-4797). The identification of the AmyR binding site will make it possible to insert these sequences in other promoters not normally regulated by AmyR.

MATERIALS AND METHODS

Hybridization:

Suitable hybridization conditions for determining hybridization between a nucleotide probe and an "analogous" DNA sequence of the invention may be defined as described below. The oligonucleotide probe to be used is the DNA sequence corresponding to the transcription factor encoding part of the DNA sequence shown in SEQ ID NO: 1, i.e. nucleotides 1691..2676 + 2743..3193 + 3278..3653 in SEQ ID NO: 1, or a fragment thereof, *e.g.* nucleotides 1770-1800 in SEQ ID NO: 1.

Hybridization conditions

Suitable conditions for determining hybridization between a nucleotide probe and a homologous DNA or RNA sequence involves

pre-soaking of the filter containing the DNA fragments or RNA to hybridize in 5x SSC (standard saline citrate buffer) for 10 min, and prehybridization of the filter in a solution of 5x SSC (Sambrook, *et al.*, *supra*), 5x Denhardt's solution (Sambrook, *et al.*, *supra*), 0.5 % SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook, *et al.*, *supra*), followed by hybridization in the same solution containing a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* **132** 6-13), ^{32}P-dATP-labeled (specific activity > 1 x 10^9 cpm/µg) probe for 12 hours at ca. 65°C. The filter is then washed two times for 30 minutes in 2x SSC, 0.5 % SDS at preferably not higher than 50°C, more preferably not higher than 55°C, more preferably not higher than 60°C, more preferably not higher than 65°C, even more preferably not higher than 70°C, especially not higher than 75°C.

Molecules to which the nucleotide probe hybridizes under these conditions are detected using a Phospho Image detector.

Immunological cross-reactivity:
Antibodies to be used in determining immunological cross-reactivity may be prepared by use of a purified transcription factor. More specifically, antiserum against the transcription factor of the invention may be raised by immunizing rabbits (or rodents) according to the procedure described by N. Axelsen et al. in: A Manual of Quantitative Immunoelectrophoresis, Blackwell Scientific Publications, 1973, Chapter 23, or A. Johnstone and R. Thorpe, Immunochemistry in Practice, Blackwell Scientific Publications, 1982 (more specifically pp. 27-31). Purified immunoglobulins may be obtained from the antisera, for example by salt precipitation ($(NH_4)_2$ SO_4), followed by dialysis and ion exchange chromatography, *e.g.* on DEAE-Sephadex. Immunochemical characterization of proteins may be done either by Outcherlony double-diffusion analysis (O. Ouchterlony in: Handbook of Experimental Immunology (D.M. Weir, ed.), Blackwell Scientific Publications, 1967, pp. 655-706), by crossed immunoelectrophoresis (N. Axelsen et al., *supra*, Chapters 3 and

4), or by rocket immunoelectrophoresis (N. Axelsen et al., *op cit.*, Chapter 2).

EXAMPLES

EXAMPLE 1

Cloning of the *amyR* transcription factor from *A. oryzae*

amyR was cloned by complementation of an *A. oryzae* mutant strain unable to express two different proteins both under control of the TAKA-amylase promoter. The mutant *A. oryzae* strain ToC879 was made by mutagenesis of a strain, SRe440, containing a lipase (HLL) encoding cDNA under control of the TAKA promoter and one copy of the TAKA-amylase gene transcribed from its own promoter.

The mutant was identified and isolated by its amylase negative (amylase$^-$) phenotype and subsequently shown to be lipase negative (lipase$^-$) as well.

The strain ToC879 contains intact copies of both expression cassettes. The amylase$^-$ phenotype makes ToC879 unable to grow on plates containing 1% cyclodextrin as the sole carbon source, while the parent strain SRe440 will grow on such plates.

ToC879 has been deposited at DSM under the name DSM No.10671.

amyR was isolated by co-transforming ToC879 with an *A. oryzae* cosmid library and an autonomously replicating pHelp1 based plasmid (D. Gems, I. L. Johnstone, and A. J. Clutterbuck (1991) *Gene* **98** 61-67) carrying the *bar* gene from *Streptomyces hygroscopicus* which confers resistance to glufosinate. The transformants were subjected to selection on plates containing cyclodextrin as the sole carbon source and screened for a concurrent reversion to the lipase$^+$ phenotype.

The transforming DNA was rescued from colonies able to grow on cyclodextrin. Subcloning resulted in the isolation of a 4.3 kb DNA fragment able to complement both phenotypes of ToC879. The gene harboured on this fragment was named *amyR*.

Construction of the pHelp1 derivativ pMT1657

A plasmid, pMT1612, was made by ligation (and subsequent transformation into *E. coli* DH5a) of the following four fragments:

i) the *E. coli* vector pToC65 (described in EP 531 372) cut with *SphI/XbaI*,

ii) a PCR fragment (containing the *A. nidulans amdS* promoter) cut with *SphI/BamHI*,

iii) a 0.5 kb *BamHI/XhoI* fragment from pBP1T (B. Staubinger *et al.*, (1992) *Fungal Genetics Newsletter* **39** 82-83) containing the *bar* gene, and

iv) a 0.7 kb *XhoI/XbaI* fragment from pIC AMG/Term (EP Application No. 87103806.3) containing the *A. niger* glucoamylase transcription terminator.

The PCR fragment containing the *amdS* promoter was made using the plasmid pMSX-6B1 (M. E. Katz *et al.*, (1990) *Mol. Gen. Genet.* **220** 373-376) as substrate DNA and the two oligonucleotides 4650 (SEQ ID NO: 4) and 4561 (SEQ ID NO: 5) as primers.

4650:	CTTGCATGCCGCCAGGACCGAGCAAG,	SEQ ID NO: 4
4651:	CTTGGATCCTCTGTGTTAGCTTATAG.	SEQ ID NO: 5

pMSX-6B1 contains an *amdS* promoter up mutation called I666.

pMT1612 was cut with *HindIII*, dephosphorylated and ligated to a 5.5 kb *HindIII* fragment from pHelp1 containing the AMA1 sequence. The resulting plasmid, pMT1657 is self-replicating in *Aspergilli* and can be selected for by increased resistance to glufosinate. pMT1657 is depicted in Fig. 1, wherein PamdS represents the amdS promoter of fragment ii) above, *bar* represents fragment iii) above, and Tamg represents fragment iv) above.

Construction of the cosmid library

A cosmid library of *Aspergillus oryzae* was constructed essentially according to the instructions from the supplier of the

"SuperCos1 cosmid vector kit" (Stratagene Cloning Systems, La Jolla CA, USA).

Genomic DNA of *A. oryzae* IFO4177 was prepared from protoplasts made by standard procedures (Christensen, T., *et. al.* (1988) *Biotechnology* 6 1419-1422).

After isolation the protoplasts were pelleted by centrifugation at 2500 rpm for 5 minutes in a Labofuge T (Heto); the pellet was then suspended in 10 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 µg/ml proteinase K and 0.5% SDS as stated in the manual from the Supercos 1 cosmid vector kit; the rest of the DNA preparation was done according to the instructions of the kit.

The size of the genomic DNA was analysed by electrophoresis using the CHEF-gel apparatus (Bio-Rad Laboratories, Hercules CA, USA). A 1% agarose gel was run for 20 hours at 200 volts with a 10-50 second pulse. The gel was stained with ethidium bromide and photographed. The DNA was 50->100 kb in size. The DNA was partially digested using *Sau3A*. The size of the digested DNA was 20-50 kb determined by the same type of CHEF-gel analysis as above. The CsCl gradient banded SuperCos1 vector was prepared according to the manual. Ligation and packaging were likewise performed as described in the manual.

After titration of the library, all of the packaging mix from one ligation and packaging was transfected into the host cells, XL1-Blue MR, and plated on 50 µg/ml ampicillin LB plates. Approximately 3800 colonies were obtained. Cosmid preparations from 10 colonies showed that they all had inserts of the expected size. The colonies were picked individually and inoculated in microtiter plate wells with 100 µl LB (100 µg/ml ampicillin) and incubated at 37°C overnight. 100 µl of 50% glycerol was added to each well, and the entire library was frozen at -80°C. A total of 3822 colonies were stored.

This represents the *A. oryzae* genome approximately 4.4 times. After picking the colonies the plates were scraped off, the scrape-off pooled and the total library was also stored in four pools as frozen glycerol stock. The four pools were named ToC901-ToC904.

The individually frozen colonies in the library were inoculated onto LB-plates (100 µg/ml ampicillin) by using a multipin device of 6 rows of 8 pins fitting into half a microtiter dish. Plates were made containing colonies from all clones in the library.

The plates were incubated at 37°C overnight. Sterilized Whatman 540 filters cut to the size of a petri dish were placed upon the colonies which were incubated for two more hours at 37°C. The filters were transferred to LB plates containing 200 µg/ml of chloramphenicol and the plates were incubated overnight at 37°C.

The next day the filters were washed twice in 0.5 M NaOH for 5 minutes, then twice in 0.5 M Tris-HCl (pH7.4) for 5 minutes and then twice in 2x SSC for 5 minutes. The filters were wetted with ethanol and air dried.

Selection of *amyR* clones

Cosmid DNA was prepared from ToC901-904 and introduced into ToC879 by co-transformation with pMT1657. The transformation procedure is described in EP Application No. 87103806.3. Approximately 8700 transformants were selected by resistance to 1 mg/ml glufosinate in minimal plates (Cove D.J. (1966) *BBA* **113** 51-56) containing 1 M sucrose for osmotic stabilization and 10 mM $(NH_4)_2SO_4$.

Ten randomly chosen transformants were reisolated once on the same type of plates. Conidiospores from these 10 transformants were inoculated in minimal medium containing 1 mg/ml glufosinate and grown at 30°C until enough mycelium for DNA preparation could be harvested. DNA was prepared as described in T. Christensen *et al.* (*supra*).

The uncut DNA was applied to a 0.7% agarose gel, and electrophoresis was performed, followed by Southern blotting. The blot was hybridized with a ^{32}P-labelled SuperCos1 specific DNA fragment. Each of the ten transformants showed a band with a higher mobility than the linear chromosomal DNA. Each of the bands also hybridized to a pHelp1 specific probe, indicating that the co-transformation frequency of the cosmid library was close to 100% and that the cosmids had integrated into the autonomously replication vector pHelp1.

The transformants were unstable as expected for pHelp1 transformants. Less than 10% of the conidiospores from a glufosinate resistant colony gave rise to glufosinate-resistant progeny.

Conidiospores from all the transformants were collected in 8 pools and plated on minimal plates (Cove D.J., *supra*) containing 1 mg/ml glufosinate, 10 mM $(NH_4)_2SO_4$ and 1% b-cyclodextrin (Kleptose from Roquette Frères´, 62136 Lestem, France)

Four colonies were obtained from one of the pools and one from one of the other pools. Two of the colonies from the first pool were reisolated once on the same kind of plates.

Conidiospores from the reisolated colonies were plated on minimal plates with either glucose or cyclodextrin as a carbon source and on glufosinate-containing plates. The glufosinate resistance and the ability to grow on cyclodextrin were both unstable phenotypes with the same degree of instability. This indicated that the gene conferring the ability to grow on cyclodextrin was physically linked to pMT1657 in the transformants.

Colonies from the reisolation plates were cut out and were analysed by rocket immune electrophoresis (RIE) using an antibody raised against the HLL lipase. The transformants gave a clear reaction with the antibody, while ToC879 colonies grown on maltose gave no reaction. This led to the conclusion that both the expression of amylase (*i.e.*, growth on cyclodextrin) and

lipase (*i.e.* antibody binding) had been restored in these transformants. The gene responsible for this phenotype was named *amyR*.

Isolation of the *amyR* gene

In order to rescue the *amyR* gene from the amylase$^+$, lipase$^+$ transformants of ToC879, two different approaches were used successfully.

DNA was prepared from mycelium grown in minimal medium with cyclodextrin as the carbon source.

In the first approach the DNA was packaged into λ-heads using the Gigapack® II kit from Stratagene in an attempt to rescue the original cosmid out of the total DNA. The packaging reaction was incubated with XL1-Blue MR *E. coli* under the conditions specified by the kit supplier. The *E. coli* cells were plated on LB plates with 50 μg/ml ampicillin. Two colonies appeared on the plates; the cosmids they contained were identical and named ToC1012.

In the second approach the total DNA was used in an attempt to transform competent *E. coli* DH5a cells. Sixteen colonies were isolated and shown to contain six different plasmids by restriction enzyme digest. Each of the plasmids was digested with *EcoRI* and subjected to Southern analysis. A ^{32}P-labelled probe of a mixture of pMT1657 and SuperCos1 was used to identify DNA fragments not part of any of these vectors. Two *EcoRI* fragments, approximately 0.7 and 1.2 kb in size, did not hybridize to any of these probes. The 1.2 kb fragment was isolated, labelled with ^{32}P and used as a probe in a hybridization experiment with the filters containing the part of the cosmid library that gave rise to the original transformants. Six cosmids from the pool (ToC904), containing approximately 1000 clones did hybridize.

Of these, some were shown by restriction enzyme digestion to be identical, resulting in the isolation of four different cosmids.

All cosmids contained at least parts of the TAKA-amylase gene as well. The four cosmids and the cosmid ToC1012 were transformed into ToC879 by co-transformation with pMT1623, a pUC based plasmid that carries the *bar* gene under the control of the *A. oryzae tpi* promoter. Fifteen transformants from each co-transformation were isolated by resistance to glufosinate and tested for the ability to grow on cyclodextrin.

Eight transformants of ToC1012 and three transformants of one of the other cosmids, 41B12, were able to grow. None of the transformants of the other cosmids grew. That not all of the transformants of ToC1012 and 41B12 were able to grow is likely to be a reflection of the co-transformation frequency in each experiment. Colonies from the transformants growing on cyclodextrin were analysed by RIE, and showed that they all produced lipase.

DNA fragments obtained by digesting 41B12 with either *BglII*, *HindIII* or *PstI* were cloned into pUC19 in order to subclone *amyR* from the cosmid. The subclones were transformed into ToC879 and the transformants analysed for the ability to grow on cyclodextrin and produce lipase as described above. As depicted in Fig. 2, one plasmid called pToC316 was shown to contain an approximate 9 kb *HindIII* fragment which was identified as containing *amyR*.

Further subcloning resulted in a plasmid called pToC320 containing a 4.3 kb *HindIII/SacI* fragment, which is shown in Fig. 3 and was subsequently sequenced on an ABI DNA sequencer using both further subcloning and primer walking.

A DNA sequence of 3980 bp including the *amyR* gene is shown in SEQ ID NO: 1. The deduced amino acid sequence is shown in SEQ ID NO: 3 and reveals a Gal 4-type zinc finger sequence between amino acids 28-54. Such sequences are known to bind to DNA (Reece, R.J., and Ptashne, M. (1993) Science **261** 909-910).

amyR maps close to one of the three amylase genes in IFO4177, since it was isolated from a cosmid also containing amylase-specific DNA fragments. Mapping of the cosmid showed that the α-amylase gene and *amyR* are 5-6 kb apart. Southern analysis of genomic DNA showed that only one copy of *amyR* is present in IFO4177, and confirmed that it maps close to one of the amylase genes.

Analysis of *amyR* cDNA

mRNA was made by the method of Wahleithner, J. A., *et al.* (1996, *Curr. Genet.* **29** 395-403) from a culture of *A. oryzae* grown in maltose containing medium under conditions favorable for α-amylase production. Double stranded cDNA was made by standard procedures and used for PCR reactions with the following primers:

oligodT primer: $TTTTGTAAGCT_{31}$ SEQ ID NO. 9

23087: CCCCAAGCTTCGCCGTCTGCGCTGCTGCCG SEQ ID NO. 6

20865: CGGAATTCATCAACCTCATCAACGTCTTC SEQ ID NO. 7

20866: CGGAATTCATCGGCGAGATAGTATCCTAT SEQ ID NO. 8

A PCR reaction with the primers 20866 and 23087 resulted in a fragment of approximately 1.1 kb. The fragment was digested with *EcoRI* and *HindIII*; these restriction sites were incorporated into the primers, and cloned into a pUC19 vector cut with the same enzymes.

The insert in the resulting plasmid was sequenced, the result located one intron in this part of the gene. The intron is indicated in SEQ ID NO: 2.

Another PCR reaction with the oligodT primer and primer 20866 did not result in a distinct fragment. An aliquot of this reaction was used as the starting point for a new reaction with the oligodT primer and the primer 20865, which resulted in a fragment of approximately 1.1 kb. This fragment was digested with *EcoRI* and *HindIII* and cloned into pUC19.

Sequencing showed that the fragment contained the 3' part of *amyR* and another intron was located. This is also indicated in SEQ ID NO: 2. Three independent plasmids were sequenced at the 3' end and two polyA addition sites were located, one at bp no. 3827 and one at bp no. 3927.

EXAMPLE 2

Quantification of glucoamylase synthesis in an *amyR*⁻ strain

A. oryzae produces a glucoamylase, encoded by the *glaA* gene, which is regulated by the same substances as α-amylase (Y. Hata *et al.*(1992) *Curr. Genet.* **22** 85-91). In order to see whether *amyR* is also involved in regulation of *glaA* the synthesis of glucoamylase was measured under inducing conditions in the *amyR*⁻ strain ToC879 and in the *amyR wt* strain SRe440, from which ToC879 was directly derived.

Conidiospores from each strain were inoculated in 10 ml YPM (YP containing 2% maltose) and grown for four days at 30°C. Supernatants were collected and analysed for glucoamylase content by incubation with p-nitrophenyl a-D-glucopyranoside, a substrate that turns yellow when cleaved by glucoamylase. In the procedure used, 0.5 ml of fermentation broth was mixed with 1 ml of 0.1 M Na-acetate pH = 4.3, containing 1 mg/ml of the substrate. The samples were incubated for 3 hours at room temperature and 1.5 ml of 0.1 M $Na_2B_4O_7$ was added. The yellow colour was measured in a spectrophotometer at 400 nm. Control samples were made by mixing the supernatants first with the borate and then with the substrate solution. The results were:

	reaction-control (OD units)
SRe440	0.655
ToC879	0.000

The absence of any OD reading in the sample taken from ToC879 clearly indicate that synthesis of glucoamylase of *A. oryzae* requires the expression of the AmyR transcription factor.

EXAMPLE 3

Overexpression of AmyR

A plasmid, pToC342, containing the coding region and 3' noncoding sequences of *amyR* fused to the promoter for the *A. oryzae tpi* gene was constructed. The *tpi* gene codes for triosephosphate isomerase, a constitutively expressed enzyme involved in primary metabolism. The *A. oryzae tpi* gene was isolated by crosshybridization with an *A. nidulans* cDNA clone according to the procedure of McKnight, G.L., *et al*, (1986, Cell 46 143-147). Sequencing led to identification of the structural gene. The promoter used was a fragment of approximately 700bp immediately upstream of the coding region. pToC342 was able to complement the mutation in ToC879. To pToC342 was further added the *A. oryzae pyrG* gene and the resulting plasmid, pToC359, was transformed into JaL250, a *pyrG* mutant of JaL228 described in patent application DK1024/96 filed 1996-09-19. Strains containing multiple copies of pToC359 were found to synthesise increased levels of glucoamylase.

Construction of pToC342 and pToC359

A PCR reaction was made with pToC320 as the template and the following primers:

8753 GTTTCGAGTATGTGGATTCC
8997 CGGAATTCGGATCCGAGCATGTCTCATTCTC

The resulting fragment was cut with *EcoRI/ApaI* to produce a fragment of approximately 180bp which was then cloned into pToC320 that had been digested with *EcoRI/ApaI*. The resulting plasmid, pToC336, was sequenced to confirm that the PCR fragment was intact. The 2.6kb *BamHI/SacI* fragment of pToC336 containing the coding region and the 3' untranslated sequence of *amyR* and an *EcoRI/BamHI* fragment of approximately 700bp containing the *tpi* promoter was cloned into *EcoRI/SacI* digested pUC19. The *BamHI* site downstream of the *tpi* promoter was introduced *in vitro*, whereas the *EcoRI* site is an endogenous site from the original *tpi* clone. The resulting plasmid, called pToC342, was

cut with *HindIII*, dephosphorylated and ligated to a 1.8 kb *HindIII* fragment containing the *A. oryzae pyrG* gene, resulting in a plasmid which was called pToC359. The structure of both pToC342 and pToC359 are shown in Fig. 4, wherein Ptpi represents the *tpi* promoter and TamyR represents the 3' noncoding region of *amyR*. The cloning of the *pyrG* gene has been previously described in WO 95/35385.

Expression in A. oryzae JaL250

JaL250 is a *pyrG* mutant of JaL228 selected by resistance to 5-fluoro-orotic acid. JaL228 has been described in patent application DK1024/96 filed 1996-09-19. JaL250 was transformed with pToC359 using standard procedures and by selecting for relief of uridine requirement. The transformants were reisolated twice through conidiospores and grown for four days in YP + 2% maltose at 30°C. Secreted glucoamylase was measured by the ability to cleave p-nitrophenyl a-D-gluco-pyranoside. The transformants had 5-31 arbitrary glucoamylase units/ml in the fermentation broth, while JaL228 had 2-3 units/ml. The best transformant was named ToC1200. Southern analysis showed that multiple copies of pToC359 had integrated into the genome of ToC1200. Because of the a-amylase promoter, ToC1200 may be used advantageously as a host strain for expression plasmids.

EXAMPLE 4

Carbon catabolite repression of the TAKA-promoter

The TAKA-amylase promoter is subject to carbon catabolite repression. In *Aspergilli* carbon catabolite repression is at least partially mediated via the transcriptional repressor CreA, a homologue to *S. cerevisiae* MIG1. The DNA binding sites in promoters under CreA control are known to be GC-rich and seemingly identical to the MIG1 sites in *S. cerevisiae*. The TAKA-amylase promoter contains several potential CreA binding sites. To determine whether this promoter is involved in carbon catabolite repression, three such sites were mutated, but provided only partial relief of carbon catabolite repression. In contrast, introduction of copies of constitutively expressed

AmyR in strains containing the modified promoter coupled to a reporter gene completely relieved repression of the reporter.

Construction of a CreA site deleted TAKA-amylase promoter

Three sites were identified as being potential CreA binding sites in the TAKA-amylase promoter by sequence comparison to known CreA and MIG1 sites. The resulting sites have the following sequences:

Site I CCCCGGTATTG
Site II CCCCGGAGTCA
Site III ATATGGCGGGT

The bases underlined were changed to A's because such changes are known to destroy MIG1 binding sites. The substitutions were made using standard site-specific mutagenesis procedures. An expression vector, pToC297, containing the modified promoter and the 3' nontranscribed sequence of the glucoamylase gene from *A. niger* was constructed. pToC297 is identical to pToC68 described in WO 91/17243 except for the changes in the promoter. Both plasmids have a unique *BamHI* site between the promoter and the terminator.

Expression of a lipase regulated by a CreA⁻ TAKA-amylase promoter

A *BamHI* fragment of approximately 950bp containing the cDNA encoding a *Humicola lanuginosa* lipase was cloned into pToC297. (The cloning and expression of the *H. lanuginosa* lipase has been previously described in EP 305 216.) The resulting plasmid, pToC298, was transformed into *A. oryzae* IFO4177 by co-transformation with the *A. nidulans amdS* gene, and its structure is shown in Fig. 5, wherein Ptaka-creA represents the CreA binding site deficient TAKA-amalyase promoter. The transformants were reisolated twice through conidiospores and one such transformant, ToC1075, which produces lipase, was chosen for further evaluation. ToC1075 and a p960 transformant of IFO4177 (previously described in EP 305 216) containing the lipase fused to the wild type TAKA-promoter were grown at 30°C in 10 ml YP

containing 2% or 10% glucose. Samples were taken daily for analysis of lipase in the fermentation broth. The lipase content was measured by rocket immune electrophoresis using a polyclonal antibody raised against purified lipase. Spent fermentation broth from *A. oryzae* IFO4177 did not react with the antibody. The glucose content of the fermentation broth was likewise measured daily using Tes-tape from Lilly.

On day one, glucose was detected in all cultures, but on day two glucose could be detected only in cultures originally containing 10%. The results of lipase production, shown in Fig. 6, indicate that the wild type promoter is repressed until glucose is no longer present. Thus, when the glucose becomes exhausted, lipase begins to accumulate. Fig. 6 also shows that the modified promoter is not as tightly regulated, as low levels of lipase are produced in the presence of glucose in the 10% glucose fermentation. Thus, there is partial glucose derepression seen in ToC1075.

Relief of carbon catabolite repression of lipase in ToC1075 by pToC342

ToC1075 was transformed with pToC342 by co-transformation with the *bar*-containing plasmid, pMT1623. Strains containing multible copies of pToC342 and which retained the lipase expression cassette were identified by Southern blot analysis; one such strain was. ToC1075 and ToC1139 were grown at 30°C in 10 ml YP containing either 2% or 10% glucose, and samples were assayed daily for lipase and glucose. The lipase was measured by cleavage of para-nitrophenyl-butyrate. The glucose content was measured with Tes-tape from Lilly. The results, shown in Fig. 7, indicate that ToC1075, as before, provides partial relief of glucose repression while lipase production by ToC1139 is independent of the presence of glucose.

EXAMPLE 5

Southern analysis of *A. niger* for the *amyR* gene

The syntheses of a-amylase and glucoamylase in *A. niger*, as in *A. oryzae*, are regulated by the carbon source. It is therefore

likely that *A. niger* also contains an *amyR* gene. This hypothesis was tested by looking for cross-hybridization between the *A. oryzae amyR* gene and *A. niger* chromosomal DNA.

DNA was prepared from *A. niger* by conventional methods. The DNA was cut with *BamHI*, *BglII*, *EcoRI*, *HindIII*, *SalI*, *XmaI* or *XbaI*, and the resulting DNA fragments were separated by electrophoresis on an agarose gel. The DNA was then blotted onto a nitrocellulase membrane and hybridized with a ^{32}P-labelled probe containing part of the structural *A. oryzae amyR* gene. The probe was made by PCR on pToC320 and starts at bp. no. 1683 and ends at bp. no. 2615 as shown in SEQ ID NO: 1. The hybridization was conducted in 10x Denhardt's solution, 5x SSC, 10mM EDTA, 1% SDS, 0.15 mg/ml polyA, 0.05 mg/ml yeast tRNA) at 50°C overnight. After hybridization the membrane was washed under conditions of increasing stringency and the radioactivity on the membrane analysed by a PhosphoImager. Figure 8 shows the result when the membrane had been washed in 2x SSC, 0.1%SDS at 58°C. Unique bands can be seen with several of the restriction enzymes. Thus, the *A. niger amyR* gene can be cloned on the basis of this cross-hybridization result.

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DK1024/96

WO 95/35385

WO 91/17243

EP 305 216

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: A transcription factor

(iii) NUMBER OF SEQUENCES: 9

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3980 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Aspergillus oryzae

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TCTAGACCGG CCATGTCGTG GTCCGCCAAG TTGATTCCGG ACCGTGTTGT AGTTGCTTCT 60

TTTAAGAAAC GGCACCCCTC TGCCGTCTCC GAACCGGAAT TGTAGCTAGA TGTATATGTC 120

TTGACGAACC AGGTGTCCAC GGGCAAATCC CTCACAATTG ATGGCCCGTC CCGTTCCCAT 180

CGATTTGTGC TACCTGCCGT GCAAGGCAAA ACATCCCCGT CAAACGTCCG AGGGGCATTG 240

CCTGCAATCT CTCGACCATG AGAGGGGAAG CAAGTCACGC TAGTTGCAAG GGTATAGGTC 300

CTACGCAGCA ATGAGGTGGC TTCACCCGTA CGGAGTGGGG ACAGCATGAT CAAGCCTTTT 360

GGGAACGTGA CGAAAGAGTA CCGGTTAAGC CGACGATGGG AGATGAATCT CTGCCGAGCA 420

AAGGACGAGA CCGGAAAAGA GTGTGTTGAT TCTTGGGAGC AGTTACAGTA CTTCCGTGTC 480

CGGAAATTGG AAACGTTCCT GACCAATGCT GGCGATCATC TGATATCCCT ACGCTGATTG 540

GTCCATCCCC CGATAAATGC CCGACACGAC GCTTGAGCCC TGAAAAGGTA GTATTTCTCG 600

AGAGATCCAT TCACCAGAGT CAATACTGGC AAATACATCG TTCCCCACCT CATATTCCAA 660

GGTGCCTAAA CCCCTCCGGT GTGCCGGTGA GGGTTTTCCA CGCCATCTCT AGTGGTGCCA 720

TGACGGGAGC ATCCGATGGC TTCCAGTATT GGGTGGTTGG GATGGACAAC AAGCTCCAAA 780

TAAGGGGAAT TTGCCTTTGG TCCAGGAATG AAGTCCCCGT GGGGACCAGC GGCTCAGCCC 840

AGGCTAAGAG TGGAATATCG TCATAGACCT TCGGCTCATG GGAGGTTCGG AGGTGTTACG 900

ATCCTCTTCA ATGCCATTCA TTCTCTGTTT TGACCTCGGC TTCCCGAGAG TGGTGCCTCC 960

CTTACATCCC CACATGCTGG ATGCAAGCCT GTGGTACGCT GTTTCTTTCA GAAGTAGCAG 1020

GCTAGGTTCA CGATGAGCTG CCTTTCAAAC CTGGAATAAC CATTACGTGA GACTGTTCTA 1080

CTTCTTGAAT TGATCCCTGA CTAGAGTCTG CTCTAATATG CTGTGTGGCA CGGCCGGTCC 1140

CCTCGGGGTT GCTAAGGCTG ATTTATGCAC TCCGTACAGT ATAACCCAGG GTGGCTATAG 1200

ATTCCCTGCA TCTTCCACGC TCCCTCACAA CCTGATTCCA CCATTCTTAA GCGGCCGTTA 1260

GCCTCGATGG GGTATAATGG AGTTAACTAT AAACACGACT CTACAACGAA TCCCGATGTG 1320

AGTTTGGAAC GAGTTGTTAC CGATGGGTCC TCCCATTTGT TAGGAGTGAC GCTAGGGGAC 1380

CTTTAGGGCA CAGACTAAAC CAAGACAAAG ATGGAGTAGA CTCCAGGTAG ATTAATTCCA 1440

ATCTTCTTGC CAAAGTAACG CGGGGTTTTT TGCACCTGCA GCCTCTTTTT TTTCTTTTTT 1500

CTTTTTTTTC TTTTTTTATT GTTCCCCAGA TTTCTTTTCT TTTTCTTCAA TCCTGACGTT 1560

CTCAACCGTG ATGGCGACAC AGCCCGCTTC GCTATCCCTC GCTTTTACGT CGGCCATTCT 1620

TCTAGTTGCT CTCGCGGGAT GCCATGATTT CTAAAGGCTC CACATCGGCG AGATAGTATC 1680

CTATCCGAGC ATGTCTCATT CTCCAACCGA CATTCCCTCA ACATCCGAAA AGGAAATGGA 1740

GTCAACCCCA GAAAAGCCGC CTAAACAGGC CTGCGACAAT TGCCGTCGAC GCAAAATCAA 1800

GTGTTCTAGA GAGCTTCCAT GCGACAAGTG CCAGCGTCTT CTTCTCTCCT GTTCCTACAG 1860

CGACGTGCTC CGTCGCAAGG GCCCCAAGTT CCGCACGCTC TACCCTCTCG CTCCCATCCA 1920

TCCACTCGCC TCACGACCAC GTCCTCTCAC CAAGGAATGG CTGCCCCCAA ACCCAGGGGC 1980

TTGCCATTTG GCGTCCCCGA CGTCTCCGCC GTCCACCGTA GCGGACGCCC AGTATCTACA 2040

TCCAGACTTC TCGGAGTCGT TCACTCGACT ACCACCCCCA GATCTCGTCT CCTCTCCCGA 2100

CTCGACAAAC TCGCTATTCG ACTCGTCCAC TATCGGCGCA CTCCCCGCGC CACGCCGTCT 2160

GTCGACGCCA AACCTTCTAG CCCATGTCAA TGTCTTCCTC AAGTACCTGT TCCCGATCAT 2220

GCCCGTCGTG AGACAGGACC AGCTGCAGCA GGACTGCCAC CAGCCGGAGC GCTTGTCTCC 2280

CCAACGCTAC GCTTTCATTG CCGCTCTATG CGCGGCCACG CACATCCAAC TGAAGCTGGA 2340

CGGTGCAGCA CCGGGTCCCG AGGCGGCTTC CGCGCGAGCC AGCCTCGACG GACATCCTAT 2400

GTTGTCGGGA GAAGAACTCC TGGCTGAAGC CGTGCGCGCA AGAAAGGAAT ACAACGTGGT 2460

CGACGAAATT AACATGGAAA ACCTCCTAAC CTCCTTCTTT CTCTTCGCCG CCTACGGAAA 2520

CCTAGACAGA CAGGATCAGG CCTGGTTCTA CCTATGTCAG ACCACGTCCA TGGTCTTCAC 2580

ACTAGGCCTA CAACGGGAAT CCACATACTC GAAACTAAGC GTCGAGGAAG CAGAAGAGAA 2640

AAGGAGAGTA TTCTGGCTCT TATTCGTCAC AGAAAGGTAA GAAAAGAAAA AACTCTACTT 2700

TCCCAATCAC CACCACGTAC CAAAAATAAC ACGAAAAACC AGAGGCTACG CATTACAACA 2760

AGCAAAACCA GTCATGCTCC GCAACTCCAT CCACAAACCA CAGGTCCTGT GCTCAGACGA 2820

CCCAATCCTA GCCTACGGGT TCATCAACCT CATCAACGTC TTCGAAAAGC TCAGCCCAAA 2880

TCTCTACGAC TGGGTCTCCG CCGGCGGCAG CAGCGCAGAC GGCGACCCCC CGCCTACTTC 2940

TTCTATCCAA TCCAGTCTCG CCAAGCAAAT CTCCCTGGAG GGCGTCTCCG AGATCCAGAA 3000

AGTAGACATC CTCATCACTC AGCAATGGCT ACAAACCATG ATGTGGAAAC TCTCCATGAC 3060

CCACGTCACA CAGCCCGGCT CTCGCGATGA CGCCGTTCTC CCCTTCCACC TGCCCGTGCT 3120

AGTCGGCAAG GCCGTCATGG GCGTCATCGC CGCGGCATCC CAAGGTGCTG TTGACGCTCA 3180

TGGTATCGGA ATGGTAAGAA AGCGACCTTA CCTCATCACA CCCTCCCTCA TCAGTCACTC 3240

CCCATCATCT ATACCCGCAA TCTAACAAAA ACCGCAGGAA CAAAAACTCT ACGACCTCGG 3300

CACCTCCGTA GCCGACGTCT CCCGCTCCCT AAGCACAAAA GCCGCCCACC ACCTCGCCGA 3360

ATCGACCATC GACCCCCGAG AACTCCTCTG GGGCATTCTC ACAACCCTAT CCCGAATCCG 3420

CGGTTCCCAA TCATACCTCT TCCCAGCGCT CGTCGAGCAA AGTCGAGGCA TCATCAGTTT 3480

CGACTGTTCG CTTTCCATCA GTGACTTTCT GCCTTCGTTT GGTGGGCCGC CGGCTATTAT 3540

GTGGCGGACG GGTGAATCTG GGTTTGATTT ATTGGGGATC GCGGATGATT TGCAAGAGAG 3600

GGAGAATGAG GGTGGGGAGG GGATTGTGGT GGCTGGGGAG GAGATTTCGT TTTGAGGGGG 3660

CTCTTTTCTT TTTCCTTTGT GGTGTGTTGT GTTGGGTGGT TCTGGGGGGG CGGGGGTGTA 3720

TATACGCTTG ACGATGTGCA TTGGGATTGG GGTTCCTACT GGTATATAAT ATGGATTGTT 3780

TTGTATATAG TCCGCTGGAG ACGGTGCAAT GATGTGGGGA TCAATCACTT CTTAGGACTC 3840

GGAGCACAGG GTGTCGGTTC TCGGGTTATT CTGAGTATGA GATTATATAG AATCAGTTAA 3900

TGATCATTAT TGTACATACC TTAAAGAAAG ATATGCTTGG CACCCCGATA TGACAATAGA 3960

AAACTGGTCT TCATTCTAGA 3980

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3980 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Aspergillus oryzae

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION:1691..2676

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION:2677..2742

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION:2743..3193

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION:3194..3277

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION:3278..3653

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:join(1691..2676, 2743..3193, 3278..3653)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TCTAGACCGG CCATGTCGTG GTCCGCCAAG TTGATTCCGG ACCGTGTTGT AGTTGCTTCT 60

TTTAAGAAAC GGCACCCCTC TGCCGTCTCC GAACCGGAAT TGTAGCTAGA TGTATATGTC 120

TTGACGAACC AGGTGTCCAC GGGCAAATCC CTCACAATTG ATGGCCCGTC CCGTTCCCAT 180

CGATTTGTGC TACCTGCCGT GCAAGGCAAA ACATCCCCGT CAAACGTCCG AGGGGCATTG 240

CCTGCAATCT CTCGACCATG AGAGGGGAAG CAAGTCACGC TAGTTGCAAG GGTATAGGTC 300

CTACGCAGCA ATGAGGTGGC TTCACCCGTA CGGAGTGGGG ACAGCATGAT CAAGCCTTTT 360

GGGAACGTGA CGAAAGAGTA CCGGTTAAGC CGACGATGGG AGATGAATCT CTGCCGAGCA 420

AAGGACGAGA CCGGAAAAGA GTGTGTTGAT TCTTGGGAGC AGTTACAGTA CTTCCGTGTC 480

CGGAAATTGG AAACGTTCCT GACCAATGCT GGCGATCATC TGATATCCCT ACGCTGATTG 540

GTCCATCCCC CGATAAATGC CCGACACGAC GCTTGAGCCC TGAAAAGGTA GTATTTCTCG 600

AGAGATCCAT TCACCAGAGT CAATACTGGC AAATACATCG TTCCCCACCT CATATTCCAA 660

GGTGCCTAAA CCCCTCCGGT GTGCCGGTGA GGGTTTTCCA CGCCATCTCT AGTGGTGCCA 720

TGACGGGAGC ATCCGATGGC TTCCAGTATT GGGTGGTTGG GATGGACAAC AAGCTCCAAA 780

TAAGGGGAAT TTGCCTTTGG TCCAGGAATG AAGTCCCCGT GGGGACCAGC GGCTCAGCCC 840

AGGCTAAGAG TGGAATATCG TCATAGACCT TCGGCTCATG GGAGGTTCGG AGGTGTTACG 900

ATCCTCTTCA ATGCCATTCA TTCTCTGTTT TGACCTCGGC TTCCCGAGAG TGGTGCCTCC 960

CTTACATCCC CACATGCTGG ATGCAAGCCT GTGGTACGCT GTTTCTTTCA GAAGTAGCAG 1020

GCTAGGTTCA CGATGAGCTG CCTTTCAAAC CTGGAATAAC CATTACGTGA GACTGTTCTA 1080

CTTCTTGAAT TGATCCCTGA CTAGAGTCTG CTCTAATATG CTGTGTGGCA CGGCCGGTCC 1140

CCTCGGGGTT GCTAAGGCTG ATTTATGCAC TCCGTACAGT ATAACCCAGG GTGGCTATAG 1200

ATTCCCTGCA TCTTCCACGC TCCCTCACAA CCTGATTCCA CCATTCTTAA GCGGCCGTTA 1260

GCCTCGATGG GGTATAATGG AGTTAACTAT AAACACGACT CTACAACGAA TCCCGATGTG 1320

AGTTTGGAAC GAGTTGTTAC CGATGGGTCC TCCCATTTGT TAGGAGTGAC GCTAGGGGAC 1380

CTTTAGGGCA CAGACTAAAC CAAGACAAAG ATGGAGTAGA CTCCAGGTAG ATTAATTCCA 1440

ATCTTCTTGC CAAAGTAACG CGGGGTTTTT TGCACCTGCA GCCTCTTTTT TTTCTTTTTT 1500

CTTTTTTTTC TTTTTTTATT GTTCCCCAGA TTTCTTTTCT TTTTCTTCAA TCCTGACGTT 1560

CTCAACCGTG ATGGCGACAC AGCCCGCTTC GCTATCCCTC GCTTTTACGT CGGCCATTCT 1620

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TCTAGTTGCT CTCGCGGGAT GCCATGATTT CTAAAGGCTC CACATCGGCG AGATAGTATC   1680

CTATCCGAGC ATG TCT CAT TCT CCA ACC GAC ATT CCC TCA ACA TCC GAA      1729
           Met Ser His Ser Pro Thr Asp Ile Pro Ser Thr Ser Glu
             1               5                  10

AAG GAA ATG GAG TCA ACC CCA GAA AAG CCG CCT AAA CAG GCC TGC GAC    1777
Lys Glu Met Glu Ser Thr Pro Glu Lys Pro Pro Lys Gln Ala Cys Asp
     15                  20                  25

AAT TGC CGT CGA CGC AAA ATC AAG TGT TCT AGA GAG CTT CCA TGC GAC    1825
Asn Cys Arg Arg Arg Lys Ile Lys Cys Ser Arg Glu Leu Pro Cys Asp
 30                  35                  40                  45

AAG TGC CAG CGT CTT CTT CTC TCC TGT TCC TAC AGC GAC GTG CTC CGT    1873
Lys Cys Gln Arg Leu Leu Leu Ser Cys Ser Tyr Ser Asp Val Leu Arg
                 50                  55                  60

CGC AAG GGC CCC AAG TTC CGC ACG CTC TAC CCT CTC GCT CCC ATC CAT    1921
Arg Lys Gly Pro Lys Phe Arg Thr Leu Tyr Pro Leu Ala Pro Ile His
             65                  70                  75

CCA CTC GCC TCA CGA CCA CGT CCT CTC ACC AAG GAA TGG CTG CCC CCA    1969
Pro Leu Ala Ser Arg Pro Arg Pro Leu Thr Lys Glu Trp Leu Pro Pro
         80                  85                  90

AAC CCA GGG GCT TGC CAT TTG GCG TCC CCG ACG TCT CCG CCG TCC ACC    2017
Asn Pro Gly Ala Cys His Leu Ala Ser Pro Thr Ser Pro Pro Ser Thr
     95                  100                 105

GTA GCG GAC GCC CAG TAT CTA CAT CCA GAC TTC TCG GAG TCG TTC ACT    2065
Val Ala Asp Ala Gln Tyr Leu His Pro Asp Phe Ser Glu Ser Phe Thr
110                 115                 120                 125

CGA CTA CCA CCC CCA GAT CTC GTC TCC TCT CCC GAC TCG ACA AAC TCG    2113
Arg Leu Pro Pro Pro Asp Leu Val Ser Ser Pro Asp Ser Thr Asn Ser
                130                 135                 140

CTA TTC GAC TCG TCC ACT ATC GGC GCA CTC CCC GCG CCA CGC CGT CTG    2161
Leu Phe Asp Ser Ser Thr Ile Gly Ala Leu Pro Ala Pro Arg Arg Leu
            145                 150                 155

TCG ACG CCA AAC CTT CTA GCC CAT GTC AAT GTC TTC CTC AAG TAC CTG    2209
Ser Thr Pro Asn Leu Leu Ala His Val Asn Val Phe Leu Lys Tyr Leu
        160                 165                 170

TTC CCG ATC ATG CCC GTC GTG AGA CAG GAC CAG CTG CAG CAG GAC TGC    2257
Phe Pro Ile Met Pro Val Val Arg Gln Asp Gln Leu Gln Gln Asp Cys
    175                 180                 185

CAC CAG CCG GAG CGC TTG TCT CCC CAA CGC TAC GCT TTC ATT GCC GCT    2305
His Gln Pro Glu Arg Leu Ser Pro Gln Arg Tyr Ala Phe Ile Ala Ala
190                 195                 200                 205

CTA TGC GCG GCC ACG CAC ATC CAA CTG AAG CTG GAC GGT GCA GCA CCG    2353
Leu Cys Ala Ala Thr His Ile Gln Leu Lys Leu Asp Gly Ala Ala Pro
                210                 215                 220

GGT CCC GAG GCG GCT TCC GCG CGA GCC AGC CTC GAC GGA CAT CCT ATG    2401
Gly Pro Glu Ala Ala Ser Ala Arg Ala Ser Leu Asp Gly His Pro Met
            225                 230                 235

TTG TCG GGA GAA GAA CTC CTG GCT GAA GCC GTG CGC GCA AGA AAG GAA    2449
Leu Ser Gly Glu Glu Leu Leu Ala Glu Ala Val Arg Ala Arg Lys Glu
        240                 245                 250

TAC AAC GTG GTC GAC GAA ATT AAC ATG GAA AAC CTC CTA ACC TCC TTC    2497
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Tyr Asn Val Val Asp Glu Ile Asn Met Glu Asn Leu Leu Thr Ser Phe
    255                 260                 265

TTT CTC TTC GCC GCC TAC GGA AAC CTA GAC AGA CAG GAT CAG GCC TGG      2545
Phe Leu Phe Ala Ala Tyr Gly Asn Leu Asp Arg Gln Asp Gln Ala Trp
270                 275                 280                 285

TTC TAC CTA TGT CAG ACC ACG TCC ATG GTC TTC ACA CTA GGC CTA CAA      2593
Phe Tyr Leu Cys Gln Thr Thr Ser Met Val Phe Thr Leu Gly Leu Gln
            290                 295                 300

CGG GAA TCC ACA TAC TCG AAA CTA AGC GTC GAG GAA GCA GAA GAG AAA      2641
Arg Glu Ser Thr Tyr Ser Lys Leu Ser Val Glu Glu Ala Glu Glu Lys
        305                 310                 315

AGG AGA GTA TTC TGG CTC TTA TTC GTC ACA GAA AG  GTAAGAAAAG           2686
Arg Arg Val Phe Trp Leu Leu Phe Val Thr Glu Arg
    320                 325

AAAAAACTCT ACTTTCCCAA TCACCACCAC GTACCAAAAA TAACACGAAA AACCAG A      2743

GGC TAC GCA TTA CAA CAA GCA AAA CCA GTC ATG CTC CGC AAC TCC ATC      2791
Gly Tyr Ala Leu Gln Gln Ala Lys Pro Val Met Leu Arg Asn Ser Ile
330                 335                 340                 345

CAC AAA CCA CAG GTC CTG TGC TCA GAC GAC CCA ATC CTA GCC TAC GGG      2839
His Lys Pro Gln Val Leu Cys Ser Asp Asp Pro Ile Leu Ala Tyr Gly
            350                 355                 360

TTC ATC AAC CTC ATC AAC GTC TTC GAA AAG CTC AGC CCA AAT CTC TAC      2887
Phe Ile Asn Leu Ile Asn Val Phe Glu Lys Leu Ser Pro Asn Leu Tyr
        365                 370                 375

GAC TGG GTC TCC GCC GGC GGC AGC AGC GCA GAC GGC GAC CCC CCG CCT      2935
Asp Trp Val Ser Ala Gly Gly Ser Ser Ala Asp Gly Asp Pro Pro Pro
    380                 385                 390

ACT TCT TCT ATC CAA TCC AGT CTC GCC AAG CAA ATC TCC CTC GAG GGC      2983
Thr Ser Ser Ile Gln Ser Ser Leu Ala Lys Gln Ile Ser Leu Glu Gly
395                 400                 405

GTC TCC GAG ATC CAG AAA GTA GAC ATC CTC ATC ACT CAG CAA TGG CTA      3031
Val Ser Glu Ile Gln Lys Val Asp Ile Leu Ile Thr Gln Gln Trp Leu
410                 415                 420                 425

CAA ACC ATG ATG TGG AAA CTC TCC ATG ACC CAC GTC ACA CAG CCC GGC      3079
Gln Thr Met Met Trp Lys Leu Ser Met Thr His Val Thr Gln Pro Gly
            430                 435                 440

TCT CGC GAT GAC GCC GTT CTC CCC TTC CAC CTG CCC GTG CTA GTC GGC      3127
Ser Arg Asp Asp Ala Val Leu Pro Phe His Leu Pro Val Leu Val Gly
        445                 450                 455

AAG GCC GTC ATG GGC GTC ATC GCC GCG GCA TCC CAA GGT GCT GTT GAC      3175
Lys Ala Val Met Gly Val Ile Ala Ala Ala Ser Gln Gly Ala Val Asp
    460                 465                 470

GCT CAT GGT ATC GGA ATG GTAAGAAAGC GACCTTACCT CATCACACCC             3223
Ala His Gly Ile Gly Met
475

TCCCTCATCA GTCACTCCCC ATCATCTATA CCCGCAATCT AACAAAAACC GCAG GAA      3280
                                                        Glu
                                                        480

CAA AAA CTC TAC GAC CTC GGC ACC TCC GTA GCC GAC GTC TCC CGC TCC      3328
Gln Lys Leu Tyr Asp Leu Gly Thr Ser Val Ala Asp Val Ser Arg Ser
            485                 490                 495
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CTA AGC ACA AAA GCC GCC CAC CAC CTC GCC GAA TCG ACC ATC GAC CCC      3376
Leu Ser Thr Lys Ala Ala His His Leu Ala Glu Ser Thr Ile Asp Pro
            500                 505                 510

CGA GAA CTC CTC TGG GGC ATT CTC ACA ACC CTA TCC CGA ATC CGC GGT      3424
Arg Glu Leu Leu Trp Gly Ile Leu Thr Thr Leu Ser Arg Ile Arg Gly
        515                 520                 525

TCC CAA TCA TAC CTC TTC CCA GCG CTC GTC GAG CAA AGT CGA GGC ATC      3472
Ser Gln Ser Tyr Leu Phe Pro Ala Leu Val Glu Gln Ser Arg Gly Ile
    530                 535                 540

ATC AGT TTC GAC TGT TCG CTT TCC ATC AGT GAC TTT CTG CCT TCG TTT      3520
Ile Ser Phe Asp Cys Ser Leu Ser Ile Ser Asp Phe Leu Pro Ser Phe
545                 550                 555                 560

GGT GGG CCG CCG GCT ATT ATG TGG CGG ACG GGT GAA TCT GGG TTT GAT      3568
Gly Gly Pro Pro Ala Ile Met Trp Arg Thr Gly Glu Ser Gly Phe Asp
                565                 570                 575

TTA TTG GGG ATC GCG GAT GAT TTG CAA GAG AGG GAG AAT GAG GGT GGG      3616
Leu Leu Gly Ile Ala Asp Asp Leu Gln Glu Arg Glu Asn Glu Gly Gly
            580                 585                 590

GAG GGG ATT GTG GTG GCT GGG GAG GAG ATT TCG TTT TGAGGGGGCT           3662
Glu Gly Ile Val Val Ala Gly Glu Glu Ile Ser Phe
        595                 600

CTTTTCTTTT TCCTTTGTGG TGTGTTGTGT TGGGTGGTTC TGGGGGGGCG GGGGTGTATA    3722

TACGCTTGAC GATGTGCATT GGGATTGGGG TTCCTACTGG TATATAATAT GGATTGTTTT    3782

GTATATAGTC CGCTGGAGAC GGTGCAATGA TGTGGGGATC AATCACTTCT TAGGACTCGG    3842

AGCACAGGGT GTCGGTTCTC GGGTTATTCT GAGTATGAGA TTATATAGAA TCAGTTAATG    3902

ATCATTATTG TACATACCTT AAAGAAAGAT ATGCTTGGCA CCCCGATATG ACAATAGAAA    3962

ACTGGTCTTC ATTCTAGA                                                  3980
```

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 604 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
Met Ser His Ser Pro Thr Asp Ile Pro Ser Thr Ser Glu Lys Glu Met
 1               5                  10                  15

Glu Ser Thr Pro Glu Lys Pro Pro Lys Gln Ala Cys Asp Asn Cys Arg
            20                  25                  30

Arg Arg Lys Ile Lys Cys Ser Arg Glu Leu Pro Cys Asp Lys Cys Gln
        35                  40                  45

Arg Leu Leu Leu Ser Cys Ser Tyr Ser Asp Val Leu Arg Arg Lys Gly
    50                  55                  60

Pro Lys Phe Arg Thr Leu Tyr Pro Leu Ala Pro Ile His Pro Leu Ala
65                  70                  75                  80

Ser Arg Pro Arg Pro Leu Thr Lys Glu Trp Leu Pro Pro Asn Pro Gly
                85                  90                  95
```

Ala Cys His Leu Ala Ser Pro Thr Ser Pro Pro Ser Thr Val Ala Asp
100 105 110

Ala Gln Tyr Leu His Pro Asp Phe Ser Glu Ser Phe Thr Arg Leu Pro
115 120 125

Pro Pro Asp Leu Val Ser Ser Pro Asp Ser Thr Asn Ser Leu Phe Asp
130 135 140

Ser Ser Thr Ile Gly Ala Leu Pro Ala Pro Arg Arg Leu Ser Thr Pro
145 150 155 160

Asn Leu Leu Ala His Val Asn Val Phe Leu Lys Tyr Leu Phe Pro Ile
165 170 175

Met Pro Val Val Arg Gln Asp Gln Leu Gln Gln Asp Cys His Gln Pro
180 185 190

Glu Arg Leu Ser Pro Gln Arg Tyr Ala Phe Ile Ala Ala Leu Cys Ala
195 200 205

Ala Thr His Ile Gln Leu Lys Leu Asp Gly Ala Ala Pro Gly Pro Glu
210 215 220

Ala Ala Ser Ala Arg Ala Ser Leu Asp Gly His Pro Met Leu Ser Gly
225 230 235 240

Glu Glu Leu Leu Ala Glu Ala Val Arg Ala Arg Lys Glu Tyr Asn Val
245 250 255

Val Asp Glu Ile Asn Met Glu Asn Leu Leu Thr Ser Phe Phe Leu Phe
260 265 270

Ala Ala Tyr Gly Asn Leu Asp Arg Gln Asp Gln Ala Trp Phe Tyr Leu
275 280 285

Cys Gln Thr Thr Ser Met Val Phe Thr Leu Gly Leu Gln Arg Glu Ser
290 295 300

Thr Tyr Ser Lys Leu Ser Val Glu Glu Ala Glu Glu Lys Arg Arg Val
305 310 315 320

Phe Trp Leu Leu Phe Val Thr Glu Arg Gly Tyr Ala Leu Gln Gln Ala
325 330 335

Lys Pro Val Met Leu Arg Asn Ser Ile His Lys Pro Gln Val Leu Cys
340 345 350

Ser Asp Asp Pro Ile Leu Ala Tyr Gly Phe Ile Asn Leu Ile Asn Val
355 360 365

Phe Glu Lys Leu Ser Pro Asn Leu Tyr Asp Trp Val Ser Ala Gly Gly
370 375 380

Ser Ser Ala Asp Gly Asp Pro Pro Pro Thr Ser Ser Ile Gln Ser Ser
385 390 395 400

Leu Ala Lys Gln Ile Ser Leu Glu Gly Val Ser Glu Ile Gln Lys Val
405 410 415

Asp Ile Leu Ile Thr Gln Gln Trp Leu Gln Thr Met Met Trp Lys Leu
420 425 430

Ser Met Thr His Val Thr Gln Pro Gly Ser Arg Asp Asp Ala Val Leu
435 440 445

Pro Phe His Leu Pro Val Leu Val Gly Lys Ala Val Met Gly Val Ile
450 455 460

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Ala Ala Ala Ser Gln Gly Ala Val Asp Ala His Gly Ile Gly Met Glu
465                 470                 475                 480

Gln Lys Leu Tyr Asp Leu Gly Thr Ser Val Ala Asp Val Ser Arg Ser
                485                 490                 495

Leu Ser Thr Lys Ala Ala His His Leu Ala Glu Ser Thr Ile Asp Pro
            500                 505                 510

Arg Glu Leu Leu Trp Gly Ile Leu Thr Thr Leu Ser Arg Ile Arg Gly
        515                 520                 525

Ser Gln Ser Tyr Leu Phe Pro Ala Leu Val Glu Gln Ser Arg Gly Ile
    530                 535                 540

Ile Ser Phe Asp Cys Ser Leu Ser Ile Ser Asp Phe Leu Pro Ser Phe
545                 550                 555                 560

Gly Gly Pro Pro Ala Ile Met Trp Arg Thr Gly Glu Ser Gly Phe Asp
                565                 570                 575

Leu Leu Gly Ile Ala Asp Asp Leu Gln Glu Arg Glu Asn Glu Gly Gly
            580                 585                 590

Glu Gly Ile Val Val Ala Gly Glu Glu Ile Ser Phe
        595                 600
```

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: primer 4650

(iii) HYPOTHETICAL: YES

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CTTGCATGCC GCCAGGACCG AGCAAG 26

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: primer 4651

(iii) HYPOTHETICAL: YES

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CTTGGATCCT CTGTGTTAGC TTATAG 26

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: primer

(iii) HYPOTHETICAL: YES

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CCCCAAGCTT CGCCGTCTGC GCTGCTGCCG 30

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29x base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: primer

(iii) HYPOTHETICAL: YES

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CGGAATTCAT CAACCTCATC AACGTCTTC 29

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: primer

(iii) HYPOTHETICAL: YES

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CGGAATTCAT CGGCGAGATA GTATCCTAT 29

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: primer

(iii) HYPOTHETICAL: YES

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TTTTGTAAGC TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT T 41

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 11, line 11-13

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution
DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution *(including postal code and country)*

Mascheroder Weg 1b, D-38124 Braunschweig, GERMANY

Date of deposit	Accession Number
1996-05-10	DSM 10671

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of ~~the microorganism deposited.~~

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

Form PCT/RO/134 (July 1992)

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 11, line 31-34.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution
DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution *(including postal code and country)*

Mascheroder Weg 1b, D-38124 Braunschweig, GERMANY

Date of deposit	Accession Number
1996-05-10	DSM 10666

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

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The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

Form PCT/RO/134 (July 1992)

CLAIMS

1. A transcription factor regulating the expression of an α-amylase promoter in filamentous fungus.

2. The factor of claim 1 originating from a fungus of the genus *Aspergillus*, *Trichoderma*, *Penicillium*, *Fusarium*, *Humicola*, etc.

3. The factor of claim 2 originating from the species *A. oryzae*, *A. niger*, *A. awamori*, especially *A. oryzae* IFO4177.

4. The factor of claim 3 having an amino acid sequence comprising one or more fragments of the amino acid sequence depicted as SEQ. ID. No 3.

5. A DNA construct having a DNA sequence coding for the factor of any of the claims 1 to 4.

6. The DNA sequence of claim 5 having a DNA sequence comprising one fragment or a combination of fragments of the DNA sequence depicted as SEQ ID NO:1.

7. A DNA construct comprising a DNA sequence encoding a transcription factor exhibiting activity in regulating the expression of an α-amylase promoter in a filamentous fungus, which DNA sequence comprises

a) the transcription factor encoding part of the DNA sequence cloned into plasmid pToC320 present in *E. coli* ToC1058, DSM 10666, or

b) an analogue of the DNA sequence defined in a), which

   i) is at least 60% homologous with the DNA sequence defined in a), or

   ii) hybridizes with the same nucleotide probe as the DNA sequence defined in a), or

   iii) encodes a transcription factor which is at least 50% homologous with the transcription factor

encoded by a DNA sequence comprising the DNA sequence defined in a), or

iv) encodes a transcription factor which is immunologically reactive with an antibody raised against the purified transcription factor encoded by the DNA sequence defined in a), or

v) complements the mutation in ToC879, *i.e.* makes ToC879 able to grow on cyclodextrin and produce lipase when transformed with said DNA sequence.

8. The DNA construct according to any of the claims 5 to 7, in which said DNA sequence is obtainable from a filamentous fungus.

9. The DNA construct according to claim 8, in which said filamentous fungus belongs to any of the groups Phycomycetes, Zygomycetes, Ascomycetes, Basidiomycetes and fungi imperfecti, including Hyphomycetes such as the genera *Aspergillus*, *Penicillium*, *Trichoderma*, *Fusarium* and *Humicola*, in particular a strain from *Aspergillus* sp., *and especially from A. oryzae*.

10. The DNA construct according to claim 9, in which said DNA sequence is isolated from or produced on the basis of a DNA library of an *Aspergillus oryzae* strain.

11. The DNA construct according to claim 5 to 8, in which said DNA sequence is obtainable from a yeast strain, especially of, *Saccharomyces*.

12. The DNA construct according to claim 7, in which the DNA sequence is isolated from *Eschericia coli* DSM 10666.

13. A recombinant expression vector comprising a DNA construct according to any of claims 5 to 12.

14. A cell comprising a DNA construct according to any of claims 5 to 12, or a recombinant expression vector according to claim 13.

15. The cell according to claim 14, which is a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell.

16. The cell according to claim 15, which is a strain of *Aspergillus* sp, in particular a strain of *A. niger* or *A. oryzae*.

17. The cell according to claim 15, which is a strain of *Trichoderma* sp., in particular *T. reesei*.

18. The cell according to claim 15, which is a strain of *Saccharomyces*, in particular a strain of *S. cerevisiae*.

19. A method of producing a polypeptide of interest comprising growing a cell of any of the claims 14 to 18 under conditions conducive to the production of said factor and said polypeptide of interest, and recovering said polypeptide of interest.

20. The method of claim 19, wherein said fungus is a fungus of the genus *Aspergillus*, *Trichoderma*, *Penicillium*, *Fusarium* or *Humicola*.

21. The method of claim 20, wherein said cell is of the species *A. oryzae*, *A. niger*, or *A. awamori*.

22. The method of claim 19, 20, or 21, wherein said polypeptide of interest is a medicinal polypeptide.

23. The method of claim 22, wherein said medicinal polypeptide is a growth hormone, insulin, or a blood clotting factor.

24. The method of claim 19, 20, or 21, wherein said polypeptide is an industrial enzyme.

25. The method of claim 24, wherein said industrial enzyme is a carbonyl hydrolase, carbohydrase, protease, lipase, amylase, cellulase, oxido reductase, glucoamylase, or esterase.

26. Use of a factor of any of the claims 1 to 4 for enhancing the expression of a polypeptide of interest in a filamentous fungus.

27. The use of claim 26, wherein said factor is the factor of claim 4.

28. The use of claim 27, wherein said fungus is a fungus of the genus *Aspergillus*, *Penicillium*, *Trichoderma*, *Fusarium* and *Humicola*, in particular a strain from *Aspergillus* sp., *and especially from A. oryzae* sp.

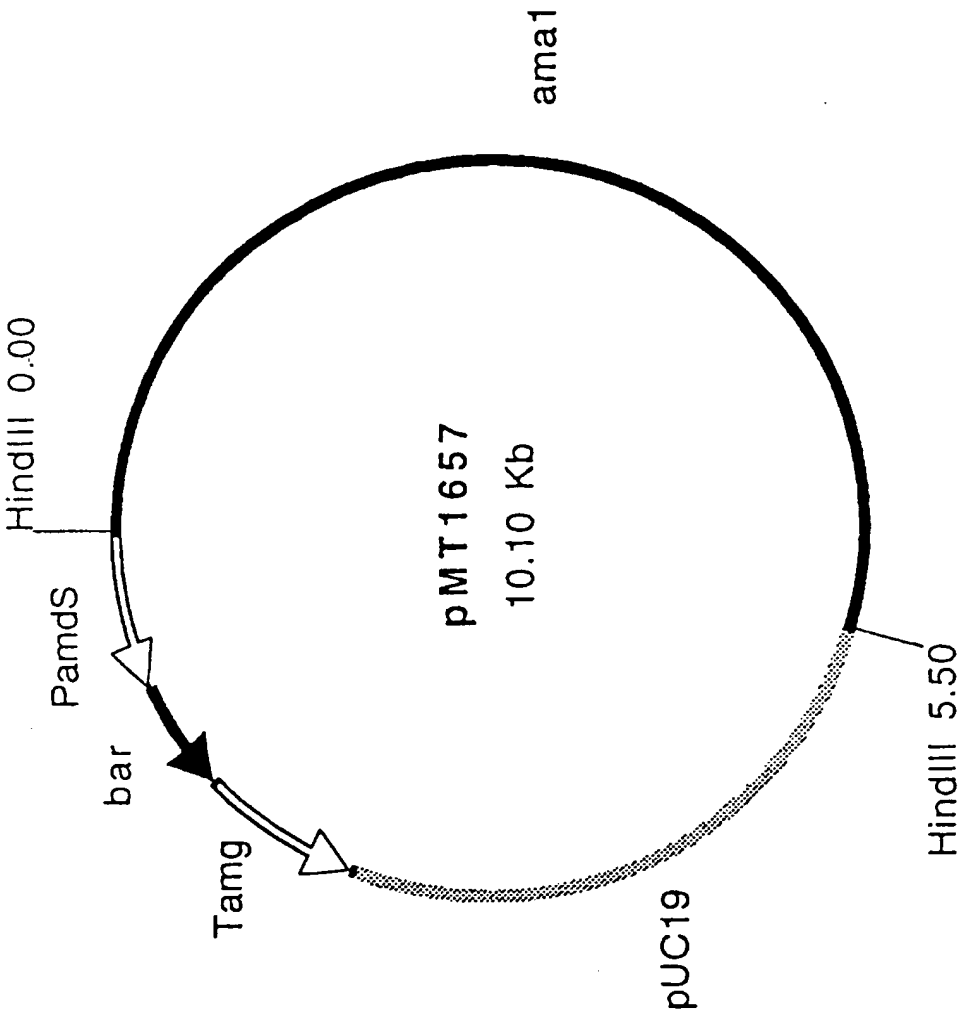
29. The use of claim 28, wherein said fungus is of the species *A. oryzae*, *A. niger*, *A. awamori*, *T. reesei*, or *T. harzianum*.

30. The use of any of the claims 26 to 29, wherein said polypeptide of interest is a medicinal polypeptide.

31. The use of claim 30, wherein said medicinal polypeptide is a growth hormone, insulin, or blood clotting factor.

32. The use of any of the claims 26 to 29, wherein said polypeptide is an industrial enzyme.

33. The use of claim 32, wherein said industrial enzyme is a carbonyl hydrolase, carbohydrase, protease, lipase, amylase, cellulase, oxido reductase, glucoamylase, or esterase.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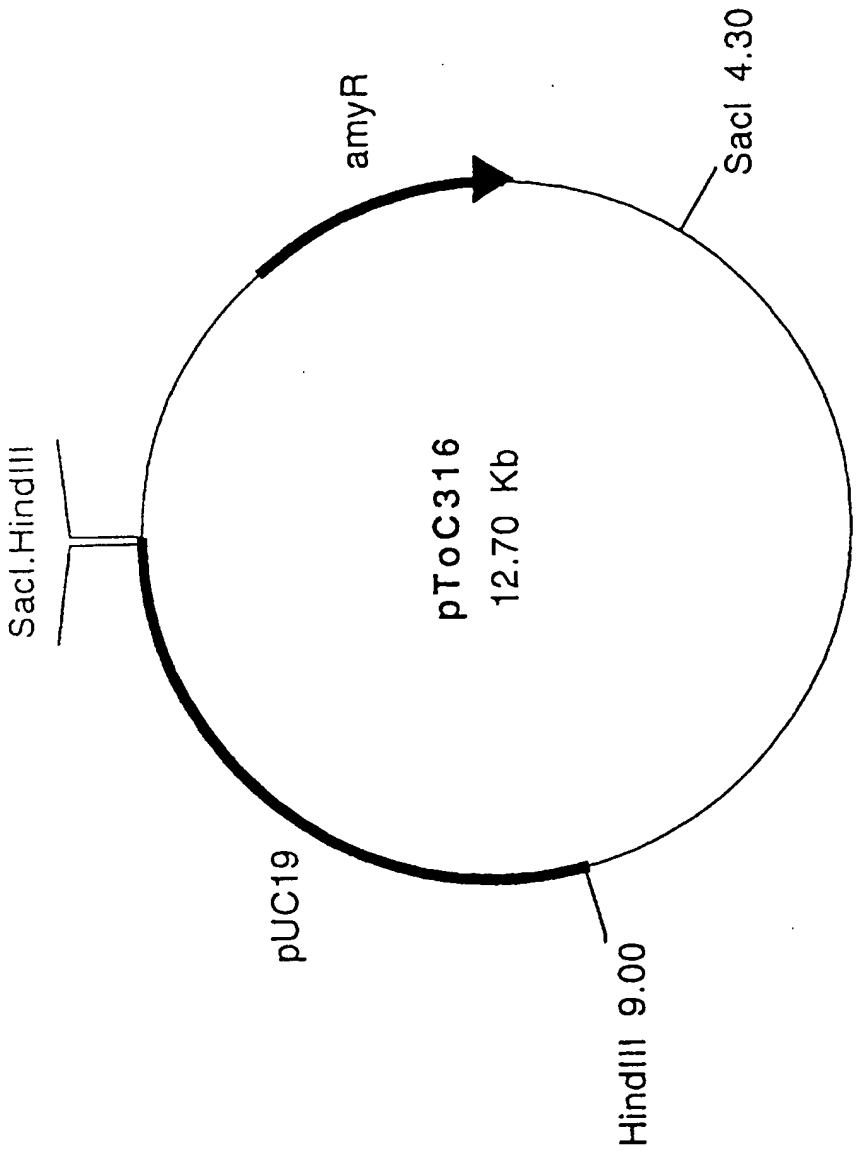
HindIII 0.00
PamdS
bar
Tamg
pMT1657
10.10 Kb
pUC19
HindIII 5.50

Fig. 1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SacI.HindIII
pToC316
12.70 Kb
pUC19
HindIII 9.00

Fig. 2

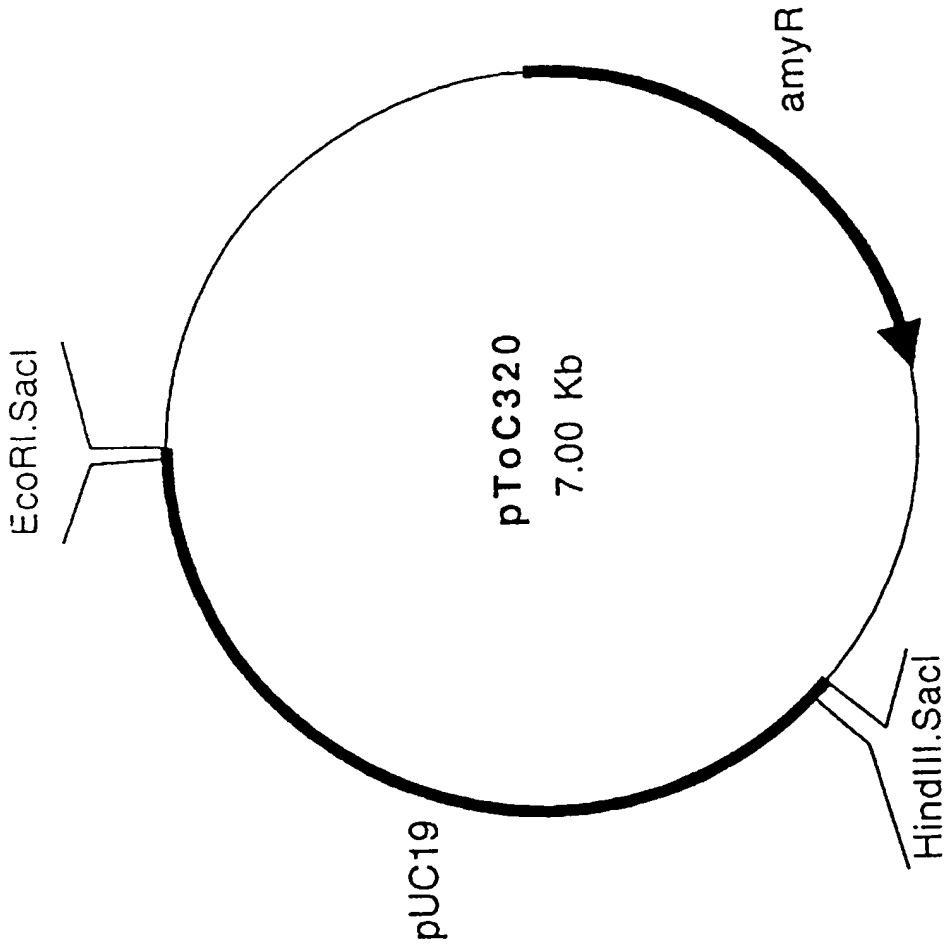

Fig. 3

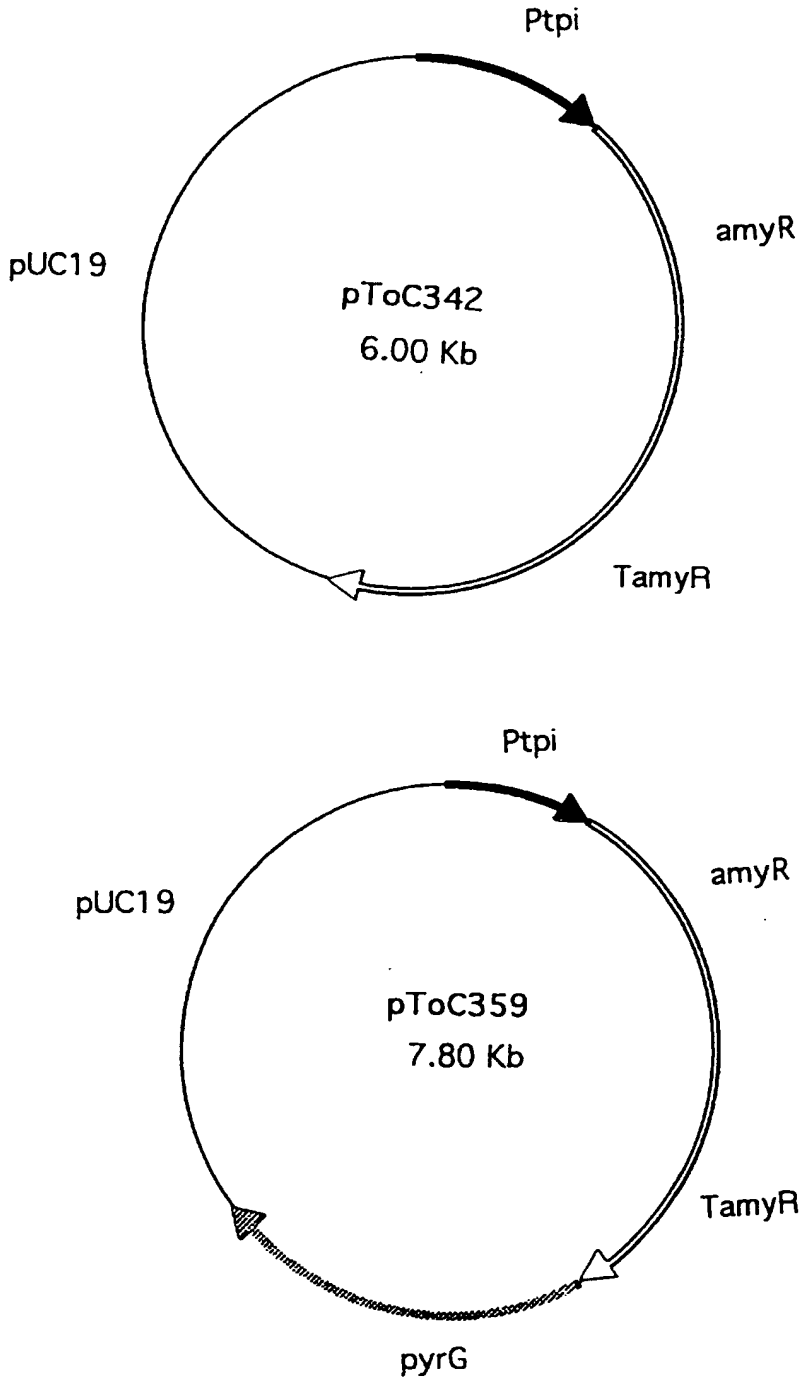
Ptpi
amyR
pUC19
pToC342
6.00 Kb
TamyR
Ptpi
amyR
pUC19
pToC359
7.80 Kb
TamyR
pyrG

Fig. 4

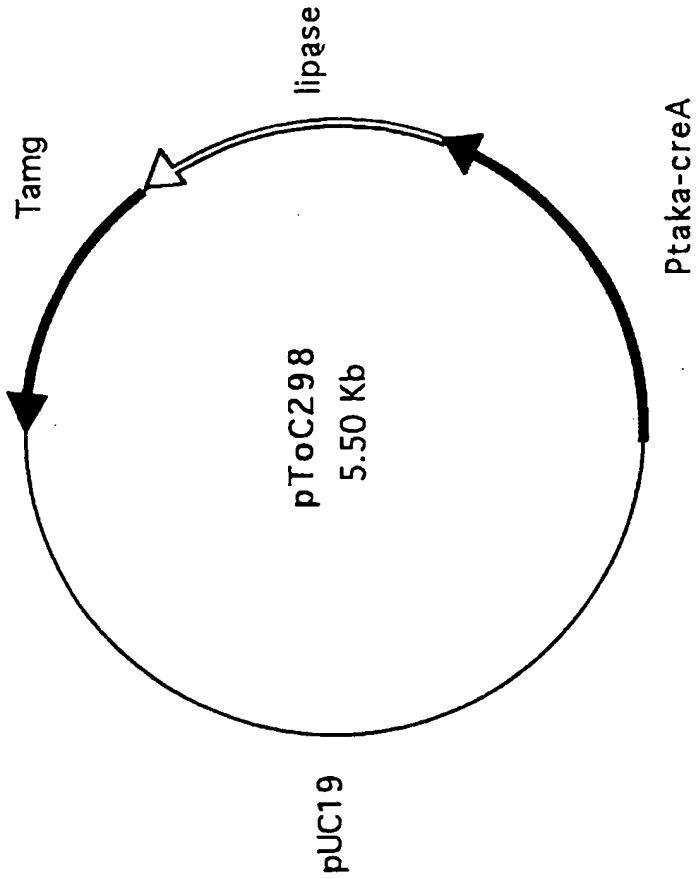

Fig. 5

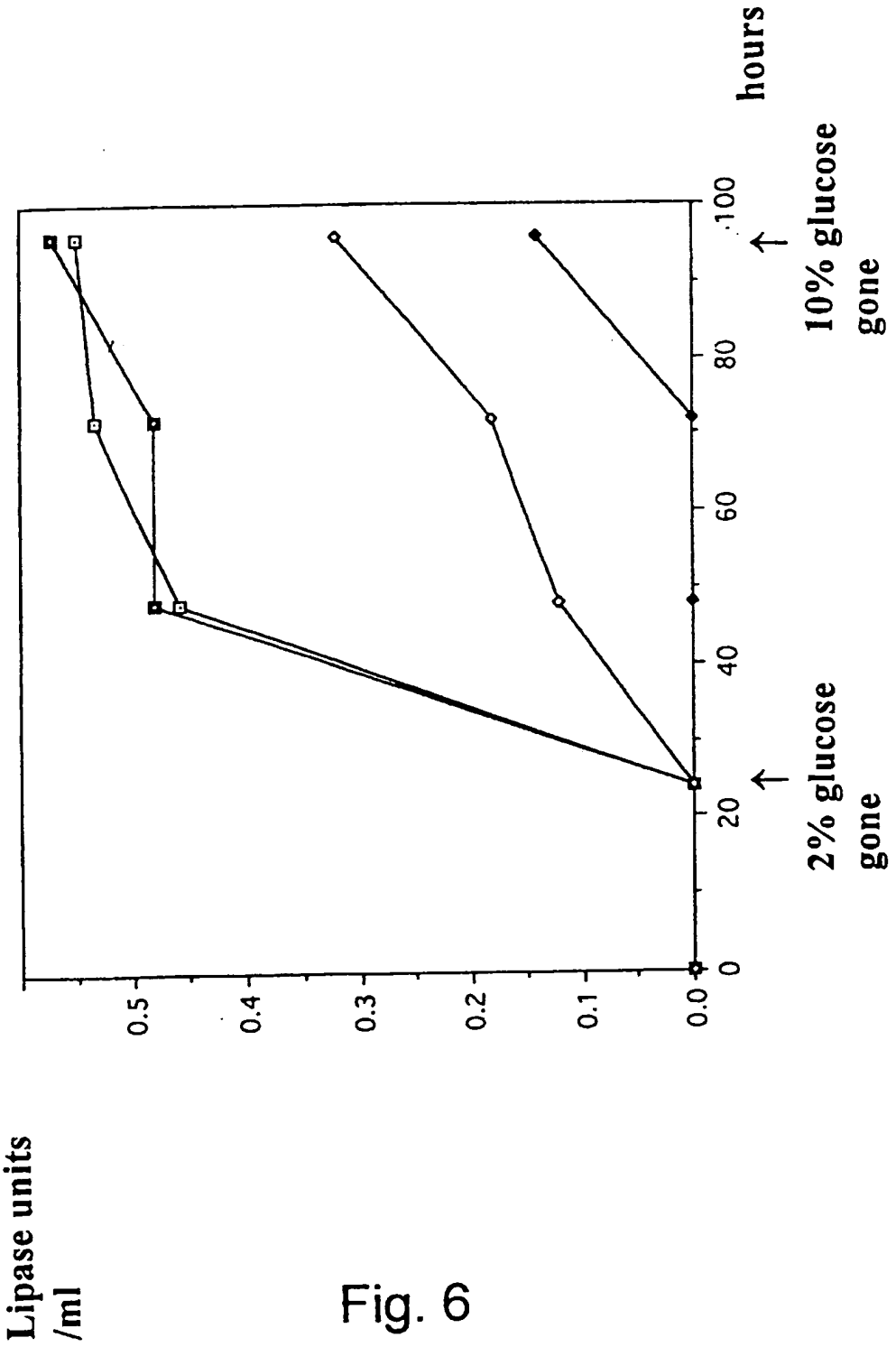
Lipase units /ml
0.0
0.1
0.2
0.3
0.4
0.5
0
20
40
60
80
100
hours
2% glucose gone
10% glucose gone

Fig. 6

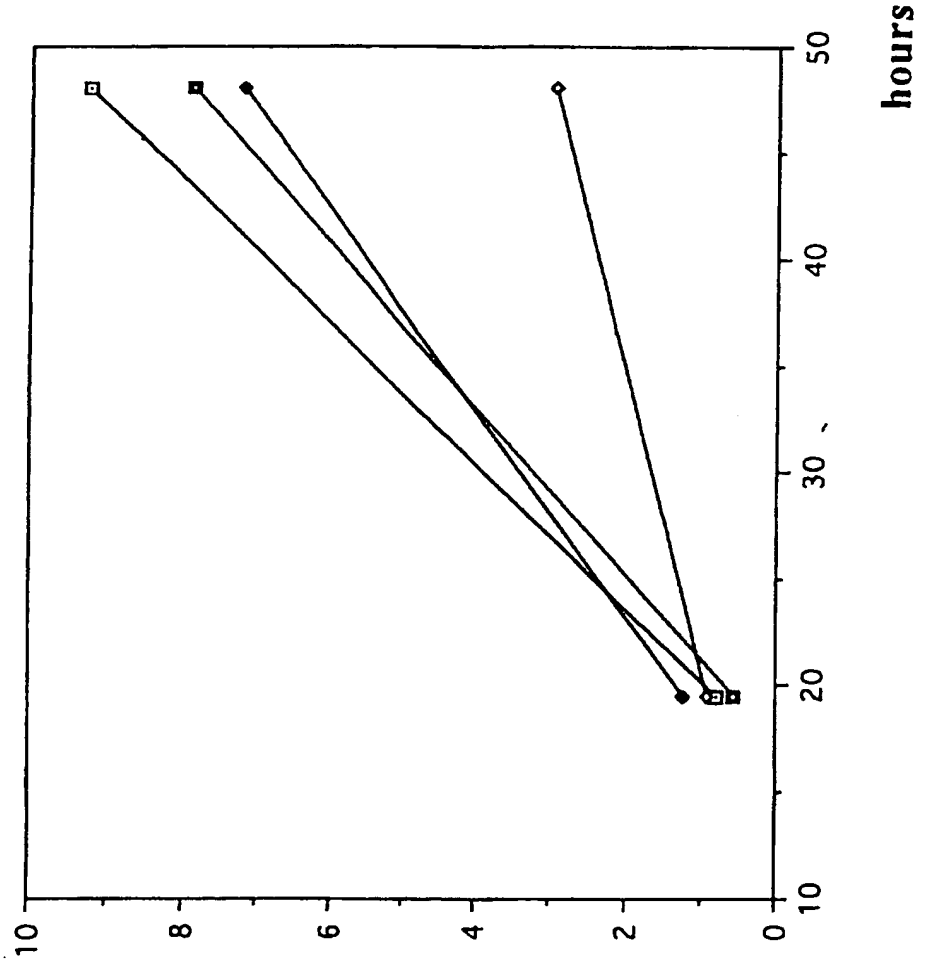

Fig. 7

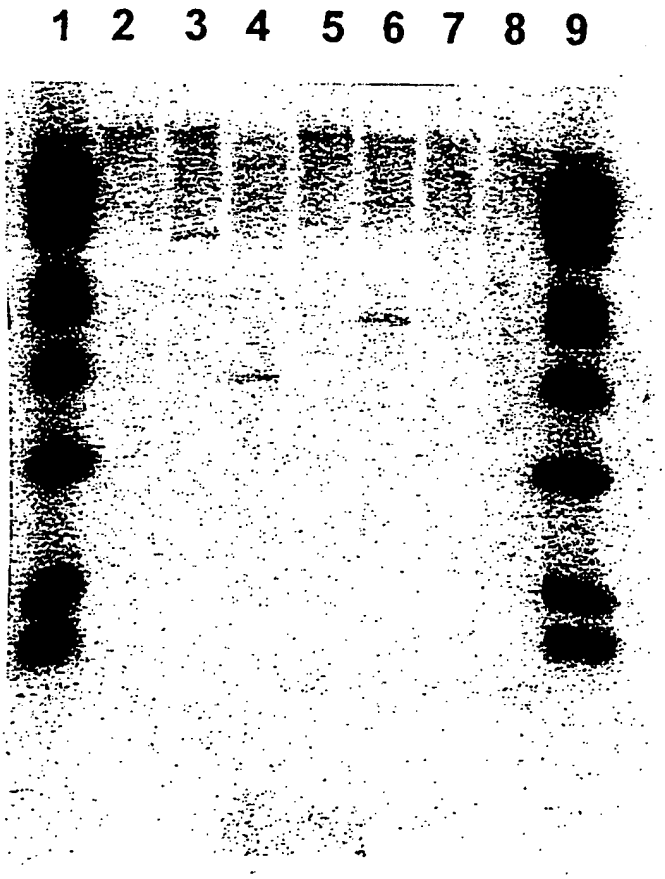

Fig. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00305

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/38, C12N 15/80, C12N 1/15
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EDOC, MEDLINE, BIOSIS, DBA, SCISEARCH GENBANK/SWISSPROT/EMBL/DDBJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Chemical Abstracts, Volume 121, No 12, 19 Sept 1994 (19.09.94), (Columbus, Ohio, USA), Verdoes, Jan C. et al, "The effect of multiple copies of the upstream region on expression of the Aspergillus niger glucoamylase en coding gene", page 272, THE ABSTRACT No 150449j, Gene 1994, 145 (2), 179-187	1-3,5,8-10, 13-18
A	--	4,6-7,11-12, 19-33

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 October 1997	07 -11- 1997
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Patrick Andersson Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00305

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Dialog Information Services, File 34, SciSearch, Dialog accession no. 13944964, Verdoes JC et al: "Molecular-Genetic Strain Improvement for the Overproduction of Fungal Proteins by Filamentous Fungi", Applied Microbiology and Biotechnology, 1995, V43, N2 (May-Jun), p 195-205 --	1-33
A	Dialog Information Service, file 154, Medline, Dialog accession no. 07510263, Medline accession no. 93204901, Nagata O. et al: "Aspergillus nidulans nuclear proteins bind to a CCAAT element and the adjacent upstream sequence in the promoter region of the starch-inducible Taka-amylase A gene", Mol Gen Genet (GERMANY) Feb 1993, 237 (1-2) p251-60 -- --------	1-33

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00305

Box I **Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1,7 and related claims
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

see next page

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00305

The wording of claim 1"A transcription factor regulating the expression of an alpha amylase promoter in filamentous fungus" is not clear as promoters are not expressed i.e. the claim does not fulfill the prescribed requirements of a claim see Art 6 and Art 17(2)(a)(ii). The claim has been interpretted as " A transcription factor regulating alpha amylase promoter initiated expression in filamentous fungus"